AMINO ACID SIDE CHAIN CONTRIBUTION TO FREE ENERGY OF TRANSFER OF TRIPEPTIDES FROM WATER TO OCTANOL

by

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To my parents

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The location of amino acids in soluble or membrane proteins is related to the hydrophobicity of the side chains. The comparison of various amino acid hydrophobicity scales reveals that they differ considerably for certain residues, especially for polar and charged residues. To obtain better estimates of amino acid hydrophobicity, the following questions were asked. (i) How closely do the hydrophobicity values derived from tripeptides agree with previous values derived from free amino acids or amino acid analogues? (ii) What are the hydrophobicity values for ionizable residues? (iii) What is the impact of using inappropriate hydrophobicity values to predict peptide partitioning into membrane or membrane spanning helices in membrane proteins?

Tripeptides of the sequence N-^{14}C-acetyl-Ala-X-Ala-NH-tButyl (AcAlaXAlaNHtButyl), where the central residue X was either Gly, Ala, Phe, Trp, Pro, His, Asp or Glu were used as model compounds. The octanol-water partition coefficients ($P_{o/w}$) were measured to determine the side chain contribution of the central residue X (Δ Gx) to the free energy of transfer.

The relative order of hydrophobicity of the side chains correlated well with previous studies. However, nonpolar amino acids such as Ala, Phe, Trp, Pro had a lower apparent hydrophobicity in the tripeptide than that in N-acetylamino acid amides. (Fauchere, J. and Pliska, V. (1983) Eur. J. Med. Chem., 18(4) 369-375). The ionizable residues Asp and Glu in the tripeptide were about 2 Kcal more hydrophilic than in the N-acetyl amino acid amides. The effect of charge on the $P_{o/w}$ of peptides was different from that of simple organic molecules. The results of the present study using blocked tripeptides (Δ Gx for the 8 amino acid side chains at pH 7.2 are 0, -0.13, -2.19, -2.52, -0.29, -0.16, 3.50 and 3.12 Kcal/mol for Gly, Ala, Phe, Trp, Pro, His, Asp and Glu, respectively) should represent a

better estimate of the hydrophobicty of amino acid side chains in peptides than previous studies because they incorporate interactions between adjacent residues.

There was a good correlation between $P_{o/w}$ and the retention time on C18 reverse phase HPLC except for the AcAlaHisAlaNHtButyl. The measurement of partitioning of tripeptides into dimyristoylphosphatidylcholine vesicles showed that the $P_{o/w}$ can give rough estimates for the membrane-water partition coefficients. Partitioning into membranes was detectable only for Phe and Trp peptides as predicted from their $P_{o/w}$.

Thermodynamic parameters were determined from the temperature dependence of partitioning. In the temperature range studied (2 °C to 65 °C) the transfer of tripeptides from water to octanol was entropy governed except for the ionized peptides. A heat capacity term was necessary to account for the transfer of tripeptides containing non polar residues as central residues whereas peptides containing Gly, His (pH 7.2) and the uncharged forms of Asp, Glu and His did not show a significant change in heat capacity.

The membrane spanning helices of two proteins, bacteriorhodopsin and bacterial photoreaction center, are predicted reasonably well with all the hydrophobicity scales used in the present comparison. A discrepancy in the prediction is detected when the assigned hydrophobicity of a residue differs by more than 10 Kcal/mol per residue. Differences of 2 or 3 Kcal/mol per residue influence the prediction only when the residues are clustered in one region.

Thesis Chairman:  Francis C. Szoka Jr. Ph. D.

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LIST OF ABBREVIATIONS

A&L	amino acid hydrophobicity scale of Abraham and Leo (1987)
AR	amino acid hydrophobicity scale of Argos and coworkers (1982)
B&B	amino acid hydrophobicity scale of Bull and Breese (1974)
Boc	butyloxycarbonyl
$CDCl_3$	chloroform-d
C_p	heat capacity
DCC	dicyclohexylcarbodiimide
DMAP	diaminomethylpyridine
DMF	dimethylformamide
DMPC	dimyristoylphosphatidylcholine
DOPC	palmitoyloleoylphosphatidylcholine
DPM	disintegration per minute
ESG	amino acid hydrophobicity scale of Engelman and coworkers (1986)
Fmoc	9-fluorenylmethyloxycarbonyl
H	enthalpy
HOBT	1-hydroxylbenzotriazole
HPLC	high pressure liquid chromatography
k'	capacity factor in HPLC
K&D	amino acid hydrophobicity scale of Kyte and Dolittle (1982)
LUV	large unilamellar vesicles
MeOD	methyl-d3 alcohol-d
MLV	multilamellar vesicles
NMM	N-methylmorpholine
RPLC	reversed phase high pressure liquid chromatography
QSAR	quantitative structure activity relationship
P	octanol-water partition coefficient on mole fraction basis
PGH	amino acid hydrophobicity scale of Parker and coworkers (1986)
$P_{o/w}$	octanol-water partition coefficient on molar basis
PRILS	amino acid hydrophobicity scale of Cornette and coworkers (1987)
S	entropy
TFA	trifuoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
WOL	amino acid hydrophobicity scale of Wolfenden and coworkers (1982)

CHAPTER 1. INTRODUCTION

1. OBJECTIVE

Amino acid side chains differ greatly in their relative affinity for water. To quantify side chain affinity for water various amino acid hydrophobicity scales have been proposed. Such hydrophobicity values are widely used to predict putative membrane spanning segments in membrane proteins (Engelman et al., 1986) or to locate the epitopes in globular proteins (Hopp and Woods, 1981, Chothia, 1984, Parker et al., 1986) in the absence of three dimensional structural information.

Membrane spanning segments are often distinctively hydrophobic whereas the surfaces of globular proteins are mostly hydrophilic. To predict such segments it is necessary to assign a proper hydrophobicity to each amino acid residue. Unfortunately the published amino acid hydrophobicity data is quite perplexing; although all hydrophobicity scales are based upon the thermodynamics of transfer of side chains from an aqueous to a non aqueous phase, there is considerable diversity among the derived hydrophobicity values. Many current hydrophobicity scales assume that the hydrophobicity of side chains in amino acids or their analogues, will remain the same when the side chain is in a peptide. This assumption may not be true.

The underlying assumption of this thesis is that better hydrophobicity values for ionized residues, proline and tryptophan need to be developed to more precisely predict lipid-peptide interactions. This assumption is based on the observations that the hydrophobicity values in the literature do not agree well, especially for proline, tryptophan and ionizable residues. The side chain contribution to the transfer free energy of peptides was determined from the octanol-water partition coefficients of a series of tripeptides and

compared to the literature values. The temperature dependence of peptide partitioning was measured to understand the thermodynamics of transfer.

2. BACKGROUND

(i) HYDROPHOBICITY

(a) Definition

'Hydrophobicity' is a measure of the relative affinity of a solute for a hydrophobic phase compared to an aqueous phase. The more hydrophobic a solute, the less it dissolves in water. It has been pointed out that 'hydrophobicity' and 'hydrophobic interaction' are two distinct concepts (Lee, 1985; Ben Naim, 1980; Pratt, 1985). Hydrophobicity measures the relative preference of the solute for the two solvents, and is described by the standard free energy of transfer of solute from water to a non aqueous solvent. 'Hydrophobic interaction' is a term to describe two or more interacting solute molecules in water such as the aggregation of nonpolar solutes in water (Figure 1). Therefore the hydrophobicity of a solute is dependent on the solvent properties not only of water but of the non-aqueous solvent whereas the hydrophobic interaction occurs in only one solvent, water.

(b) Biochemical significance

A variety of biochemical phenomena have been interpreted in terms of hydrophobic interactions or the hydrophobic effect. These include micelle formation and membrane organization (Tanford, 1980), protein folding (Chothia, 1984; Baldwin, 1986), receptor-ligand binding (Scherman et al., 1987), protein-DNA interactions (Ha et al, 1989) and

(a) HYDROPHILIC MOLECULES IN WATER

(b) NON POLAR MOLECULES IN WATER

(c) HYDROPHOBIC INTERACTION

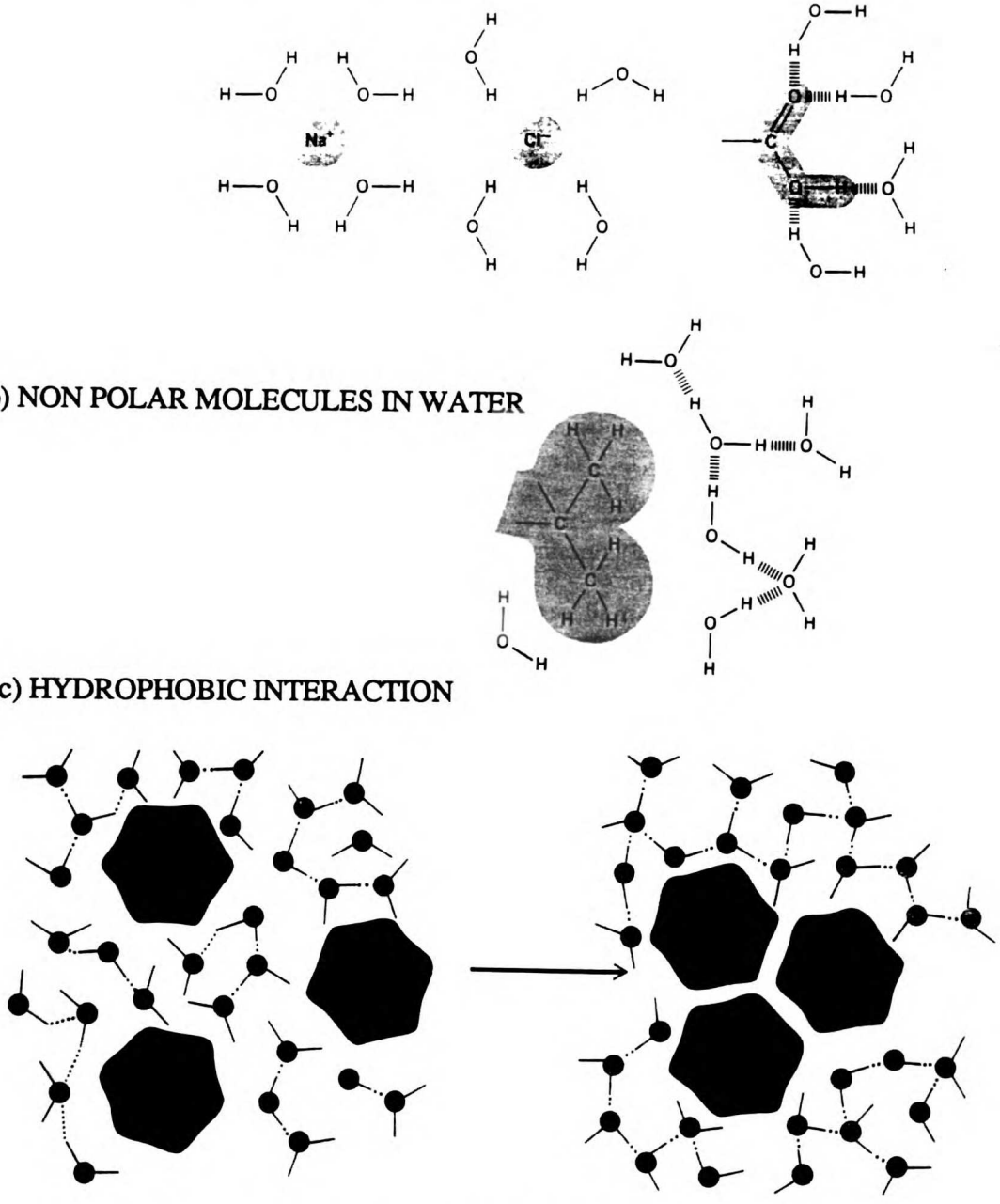


Figure 1. Solution of hydrophilic and hydrophobic molecules in water.
(a) Ions or polar molecules can interact with water molecules through ion-dipole interaction or hydrogen-bonding. (b) Non polar solutes cannot interact via hydrogen bonds with water. The water molecules form a cavity around the solute. In the process they rearrange to regenerate the hydrogen bonds disrupted by cavity formation. The entropy of the system decreases. (c) Therefore non polar molecules in water tend to aggregate to minimize the surface area in contact with water (hydrophobic interaction). Adapted from Alberts et al. (1983).

lipid-protein interactions (Boggs, J.M.,1983; Engelman et al., 1986; Engelman and Steitz, 1981; Segrest and Feldman, 1974). Non covalent interactions between molecules in aqueous solution are involved in all of these processes. The hydrophobicity of individual amino acids play an important role for processes such as protein folding, lipid-protein interactions, enzyme-substrate interactions. Details of amino acid hydrophobicity will be described later.

When a hydrophobic interaction involves intramolecular rearrangements in the aqueous phase such as protein folding, it might be difficult to justify using transfer data in quantitative studies (Lee, 1985). Pairwise interactions between two isolated side chains (Nemethy and Scheraga, 1962) might be better estimates. In reality, however, transfer data of small molecules are used to study the structure of proteins assuming the solvent property of the protein interior is similar to the solvent used in the transfer study. The solvent which best mimics the interior of protein is still controversial. When lipid protein interactions are described as the partitioning of proteins into the membrane bilayer, the question again is which solvent better mimics the lipid bilayer.

(c) Physical origin of hydrophobicity

The physical origin of hydrophobicity of non polar solutes is usually explained by the unique structure of water (Tanford, 1980; BenNaim, 1980; Lee, 1985). Frank and Evans (1945) proposed the most favored theory; water molecules become immobilized around non-polar solutes in water. This "iceberg" explanation has been well summarized by Tanford (Tanford, 1980). Briefly, when a solute is dissolved in water, a cavity is formed to accommodate the solute (Figure 1). The water molecules at the surface of the cavity rearrange themselves and regenerate the hydrogen bonds disrupted by the cavity formation. These water molecules have a restricted mobility. By doing so local order increases and entropy of the system decreases. Transfer of nonpolar or amphiphilic molecules such as

aliphatic alcohols from hydrocarbons to water is associated with negative enthalpies and entropies. The free energy of transfer is positive resulting from a negative entropy change for the process; because of this, apolar molecules tend to be expelled from water.

If the origin of hydrophobicity is structural organization of water around the hydrophobic solutes, the hydrophobicity of a solute should depend upon the size of the cavity needed to accommodate the solute in water. The incremental increase of free energy of transfer of normal saturated aliphatic hydrocarbons into water as the carbon chain length increases supports this idea (Figure 2). The size of solute can be expressed as surface area or molar volume. For saturated hydrocarbons, the free energy of transfer from a hydrocarbon solvent to an aqueous medium was estimated to be between 20 and 25 cal/mol per $Å^2$ at 25 °C (Reynolds et al., 1974). Leo and coworkers (Leo et al., 1976) showed that hydrophobicity of two different classes of solutes, expressed as octanol-water partition coefficient, were linearly dependent on the molar volume of solutes. The transfer parameters for the two classes depended on the solute property, that is, whether the surface of the solutes was rich in protons or electrons.

(d) Thermodynamics of hydrophobic interactions

When energy is transfered to a system as heat (dq) its temperature may increase (dT). The heat capacity is defined as $C = dq/dT$. When C is large, the system has a large capacity for heat and the temperature increase for a given amount of heat is smaller than that in a system with a small heat capacity. At constant pressure, with no work other than a volume change, $dH = (dq)_p$. Therefore, the heat capacity (C_p) at constant pressure is defined

$C_p = (dH/dT)_p$.

The most distinguishing feature of hydrophobic interaction is a large anomalous heat capacity change (ΔC_p) (Tanford, 1970; Baldwin, 1986; Ha et al., 1989; Dill, 1990). Frank

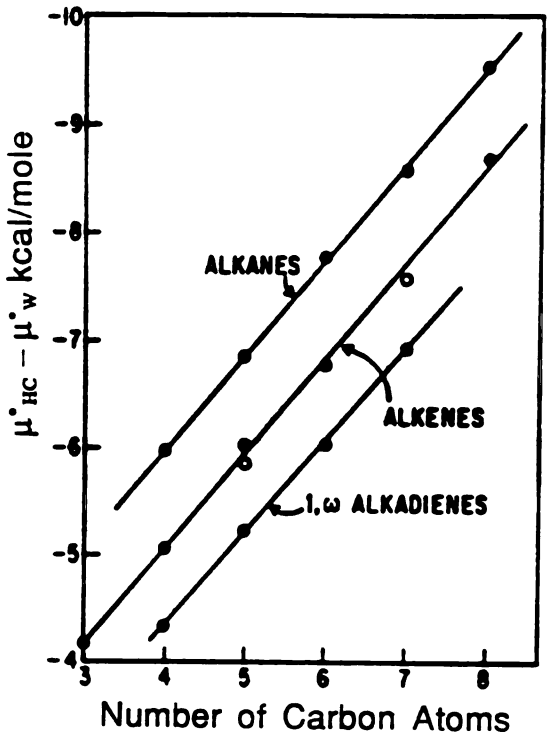


Figure 2. Free energy of transfer of hydrocarbons from aqueous solution to pure liquid hydrocarbon at 25 °C, based on solubility measurements of McAuliffe (1966). Taken from Tanford (1980).

and Evans (1945) attributed this to the excess heat needed to melt the icelike water molecules around the nonpolar solutes (Edelhoch and Osborne, 1976). Similar effects are not observed for solutions of hydrophilic compounds in water or solutions of hydrophobic compounds in organic solvents (Tanford, 1970). Therefore the transfer of hydrophobic groups from water into an organic solvent should be accompanied by a large negative ΔC_p.

As a consequence of the large heat capacity change, ΔH and ΔS are temperature dependent for the process involving a hydrophobic interaction. This has been often illustrated as curvature in the van't Hoff plot:

$$d \ln K / d T = \Delta H(T)/RT^2 \text{ or}$$

$$d \ln K / d (1/T) = -\Delta H(T)/R.$$

where K may, depending on the experiment, be the solubility or partition coefficient or some other equilibrium constant. When ΔH is independent of temperature, the plot of ln K versus 1/T will be linear and ΔH can be estimated from the slope of the plot. If ΔH is temperature dependent the plot will show a curvature. The plot of solubilities of hydrocarbons in water as a function of temperature exhibits a minimum (Figure 3). At low temperature the solubility increases as the temperature increases. However above ~18 °C, the solubility decreases as the temperature increases.

When ΔH and ΔS are functions of temperature, $\Delta H(T)$ and $\Delta S(T)$ are defined as following;

$$\Delta H(T) = \Delta H_o + \int_{T_o}^{T} \Delta C_p \, dT'$$

$$\Delta S(T) = \Delta S_o + \int_{T_o}^{T} \frac{\Delta C_p}{T'} \, dT' \quad .$$

When ΔC_p is not dependent on temperature, $\Delta C_p(T) = \Delta C_p$ and

$$\Delta H(T) = \Delta H_o + \Delta C_p(T-T_o).$$

$$\Delta S(T) = \Delta S_o + \Delta C_p \ln(T/T_o).$$

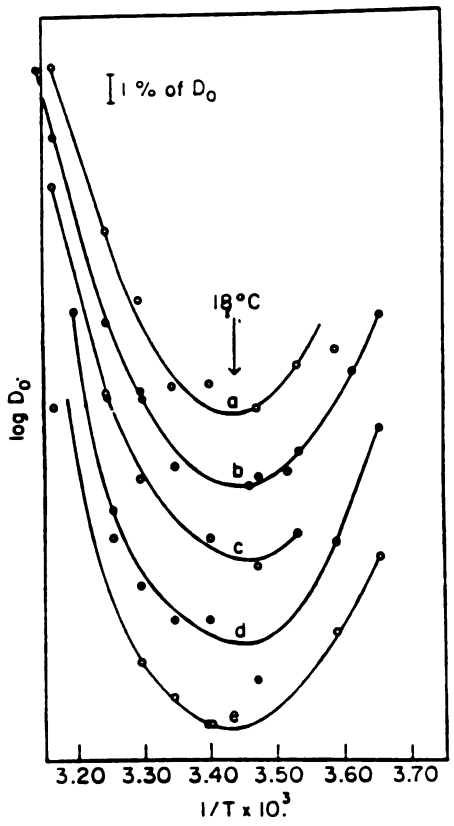


Figure 3. Logarithmic plot of solubilities of aromatic hydrocarbons in water as a function of temperature. From top to bottom: benzene, toluene, p-xylene, m-xylene, ethylbenzene. Taken from Tanford (1970).

$$\Delta G = \Delta H(T) - T\Delta S(T).$$

Therefore,

$$\Delta G = \Delta H_o + \Delta C_p (T - T_o) - T\Delta S_o - T \Delta C_p \ln(T/T_o).$$

The three parameters, ΔH, ΔS and ΔC_p can be estimated by non linear fitting of ΔG and T if experimental measurements are performed over a wide range of temperature.

(ii) SOLUTE PARTITIONING INTO VARIOUS HYDROPHOBIC PHASES

The hydrophobicity of solutes can be determined from the measurements of solubility in water (Reynolds et al., 1974) or the partition coefficients between organic solvents and water. Of the various organic solvent-water pairs, octanol-water partition coefficients have been used most frequently in Quantitative Structure Activity Relationships (QSAR) studies and a vast amount of experimental data have been documented (Leo et al, 1971). Reverse phase HPLC has been an alternative way to obtain the hydrophobicity of solutes. The difference between the two systems is shown in Figure 4. Octanol is usually considered as an isotropic bulk solvent whose property is uniform throughout the entire volume. However, local structure is possible due to the hydrogen bonding between the hydroxyl group of octanol (Figure 4-a). The distribution of water molecules (2.3 M at 25 °C) in water-saturated octanol is not known, although it is likely that waters are concentrated in the clusters of octanol hydroxyl groups (Figure 4-b). The stationary phase of reverse phase HPLC is more structured than octanol because the alkyl chains are anchored to the support material (Figure 4-c). The most complicated phase is the biomembrane which is composed of a bilayer of phospholipid containing two polar head group regions and a hydrophobic acyl chain interior (Figure 4-d). The order parameter of acyl chains varies with the distance from the head groups (Figure 4-e). Thus it is apparent that the hydrophobic phase will influence the apparent hydrophobicity of the solute.

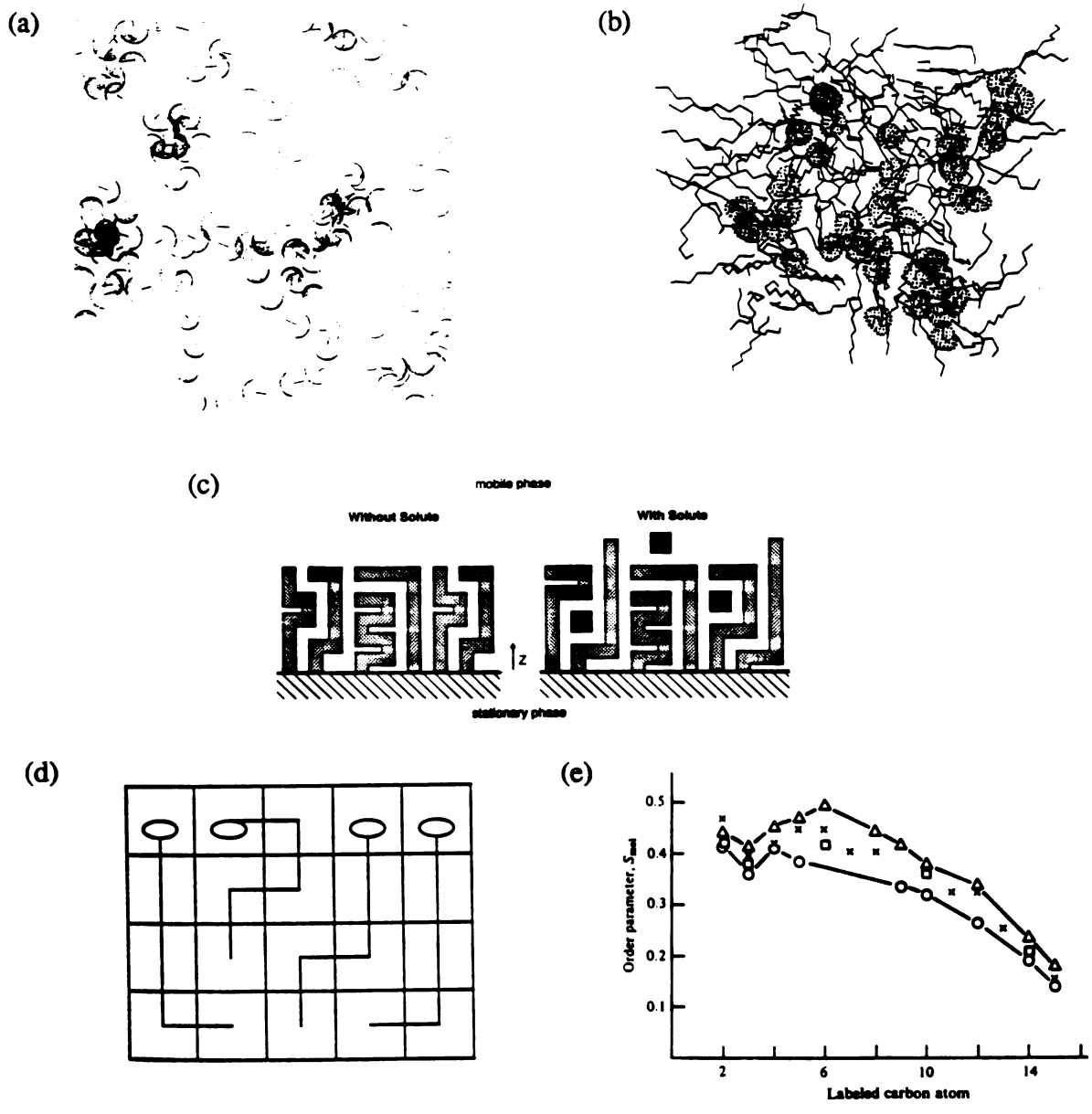


Figure 4. Solvent characteristics of various hydrophobic phases (a) Octanol is an isotropic phase. The dot-surface hemispheres represent one-half the van der Waals radius of a hydroxyl hydrogen. The straight lines represent the bond vector form the hydrogen to the hydroxyl oxygen. (b) It is likely that waters are concentrated in the clusters of octanol hydroxyl groups. Courtesy of Stephen Debolt (unpublished data). (c) Interphase model of molecular organization of stationary phase of reverse phase HPLC. Taken from Dill (1987). (d) Lattice model of bilayer acyl chain packing. Taken from Marqusee and Dill (1986). (e) Order parameter profile of various phospholipid bilayers with the chain position. Taken from Boggs (1983).

(a) Octanol-water partition coefficient

Various aspects of octanol-water partition coefficients have been reviewed (Leo et al. 1971, Davis et al. 1974, Chiou and Block, 1986). The thermodynamic background of partition coefficients and their application in structure activity relationships will be briefly described.

Definition

When two phases are in equilibrium at constant temperature and pressure, the chemical potential of a single species in both phases will be the same. The chemical potential of a solute in solvent i, μ_i and the chemical potential of the same solute in solvent j, μ_j are expressed using its mole fraction in each solvent, X_i and X_j

$$\mu_i = \mu_i^\circ + RT \ln X_i$$

$$\mu_j = \mu_j^\circ + RT \ln X_j$$

where μ_i° and μ_j° are the corresponding standard chemical potentials.

At equilibrium, $\mu_i = \mu_j$, then $\Delta \mu = 0$ so $\mu_i^\circ - \mu_j^\circ = -RT \ln X_i/Xj$.

The partition coefficient P is defined as the ratio of the equilibrium concentration of the solute in the two immiscible or partially miscible solvents, usually an organic solvent and water;

$$P = X_o/X_w$$

where X_o and X_w are the solute mole fractions at equilibrium in the organic solvent and water, respectively. Therefore the standard molar free energy of transfer of solutes from organic solvent to water can be obtained from experimental measurement of the partition coefficient:

$$\Delta G^{\circ} = \Delta\mu^{\circ} = -RT \ln P$$

It should be noted that this relationship holds only at dilute concentrations. Therefore when concentration dependence of partition coefficients are found, extrapolated value at infinite dilution should be used as partition coefficient.

When the solute concentration is expressed in units other than the mole fraction such as molarity or molality, the assumed standard state is different and also the computed value of the partition coefficient is different. Mole fraction units are favored when discussing thermodynamics because a unitary free energy change is obtained when concentration is expressed in mole fraction units. Unitary free energy reflects only the internal free energy of the solute molecule and the free energy of its contact with the surrounding solvent molecules (Tanford, 1970). If a concentration unit other than mole fraction is used, the standard free energy will include the cratic contribution in the standard state. The cratic contribution arises from the entropy of mixing of solutes and solvent and is independent of the chemical nature of solutes (Cantor and Shimmel, 1980). Therefore when two processes of different cratic contributions are considered (e.g., transfer of hydrocarbon in pure liquid to water vs. transfer of hydrocarbon in organic solvent to water) direct comparison is possible only when the unitary free energy change is known. However in most of the literature, partition coefficients are reported using the ratio of molar concentrations of solute in two phases not mole fraction. In this study conversion from one unit to the other has been done when necessary, and is indicated where appropriate.

Applications

Octanol-water was chosen as the standard reference system for many QSAR studies because pure octanol was readily available and octanol was believed to mimic the biophase better than other organic solvents (Hansch and Fujita, 1964; Fujita et al. 1964; Leo et al. 1971; Hansch and Dunn, 1972). Octanol differs from hydrocarbon solvents in that local

structure can exist in octanol due to hydrogen bonding between the hydroxyl groups of octanol (Figure 4 a, b).

The relationship between the partition coefficient and biological activity was first demonstrated by Meyer (Meyer, 1899) and Overton (Overton, 1901) who reported that narcotic activity of organic molecules were related to their oil-water partition coefficient. Octanol-water partition coefficients of model compounds have been used to calculate partition coefficients of new compounds or to estimate their biological activity. This is possible because of the linear free energy relationship and group additivity concepts.

Linear free energy relationship - Collander equation

The first extensive study of group contributions to partition coefficients was made by Collander (1954) (Davis et al., 1974). Collander measured partition coefficients of solutes between water and diethyl ether, isobutanol, octanol and oleoyl alcohol and found the following relationship, now called the Collander equation:

$$\log P_{(i)} = a \log P_{(j)} + b$$

where a and b are constants and $P_{(i)}$ and $P_{(j)}$ are partition coefficients between water and solvent i and between water and solvent j respectively. The equation shows a linear relationship of partition coefficients of a group of solutes between two different solvent systems. Therefore once the constants a and b are determined from partition coefficient measurements of a set of solutes between two solvent systems, a solute's partition coefficient in one solvent system can be deduced from that in the other solvent system. Examples of the application of this equation are presented below.

Group-additivity

The group additivity concept assumes that the free energy of transfer of a molecule is the sum of the free energy of transfer of the constitutive parts of the molecule. Dunn and collegues have reviewed (1986) different methods of estimating partition coefficient using group additivity.

The Hansch substituent constant, π, is defined as

$\pi_X = \log P_X - \log P_H$

where P_X is partition coefficient of the derivative of a parent molecule whose partition coefficient is P_H. Therefore $-RT\ 2.3\ \pi_x$ is the contribution of group X to the free energy of transfer of the molecule into octanol. Comprehensive collections of π constants have been compiled by Hansch and Leo (Hansch and Leo, 1979). When the partition coefficient of the parent compound and the π value of the substituent group are known, the partition coefficient of the derivative compound can be calculated ($\log P_X = \log P_H + \pi_X$).

Intramolecular interactions, which complicate the computation of partition coefficients caused by inductive effects, resonance effects, steric effects, branching and conformational effects, have been discussed (Leo et al. 1971). The inductive effect is observed in aromatic compounds bearing electron-withdrawing groups such as NO_2 or Cl. When lone-pair electrons are available near these electron-withdrawing groups, π values are often raised. Examples are the π values of NO_2 and Cl containing compounds. The log P values of nitrobenzene and benzene differ by -0.28 while those of 4-nitrobenzyl alcohol and benzyl alcohol differ by 0.11. Likewise the log P values of chlorobenzene and benzene differ by 0.71 while 4-chloronitrobenzene and nitrobenzene differ by only 0.54.

The resonance effect is the effect of electron delocalization on π values. Transferring a functional group from an aliphatic to an aromatic position results in an increase in lipophilicity except for NH_2. The hydrogen bonding ability of the NH_2 group with water seems to offset its resonance effect (Leo et al., 1971). The steric effect is due to the crowding of functional groups. For example when alkyl groups shield lone pair electrons, the π value is increased. On the other hand, when hydrophilic groups are crowded together

the overall hydrophilicity is reduced because fewer water molecules interact with each hydrophilic molecule than if they were separated. This is called the polar proximity effect (Abraham and Leo, 1987) as described later in this section.

Conformational effects are observed when folded conformations are possible. For example, the aqueous solubility of phenylpropyl derivatives are greater than expected. Folding of the side chain onto the phenyl ring decreases the apolar surface area so that entropy cost of solution is lowered. Both dipolar interactions and intramolecular hydrophobic interactions seem to play a role in folding. Intramolecular hydrogen bonding is another frequently observed conformational effect on π values, and is particularly important in peptides and proteins.

A newer parameter, the fragment constant f, was introduced by Nys and Rekker (1973), Rekker (1976) and Leo et al. (1975). The f constant also assumes that log P is an additive-constitutive property. Therefore log P is expressed as follows:

$$\log P = \sum a_n f_n$$

where n is the number of each type of fragment in the solute, a is the number of times a given fragment appears and f_n is the fragment value. According to this equation, calculation of log P is possible even when no "parent" value is available. The equation assumes no interaction of fragments in a given solute. Since this assumption is obviously a simplification, correction terms were included by Rekker to improve the estimation.

Although both Rekker and Leo used the same concept, their definition of the fragment was different. According to Nys and Rekker, fragments are arbitrarily selected substructural groups into which the larger structure can be decomposed. They derived the fragment constants from the multivariable regression of a data base of log P values using the above equation.

According to Leo and coworkers, a fragment is any atom or group of atoms separated by an isolating carbon. To determine the f constant, they used the following relationships between constant f and π values;

$f_{CH_3} = \log P_{CH_4} - f_H$;

$f_{CH_2} = f_{CH_3} - f_H$; and $f_{CH} = f_{CH_2} - f_H$;

$f(x) = \pi(x) + f_H$; $f_H = 1/2(\log P_{H_2})$

where $\log P_{H_2}$ is the octanol water partition coefficient of hydrogen gas.

Leo and coworkers introduced F factors in addition to f constants. The F factors are empirically derived quantities indicating an increase or decrease of hydrophobicity due to certain structural characteristics. (1) chain (F_{cBr}) or group branching (F_{gBr}) factors correct for the lowering of the partition coefficient due to the chain or group branching. (2) Bond factors (F_b) account for the decrease in log P of large chains due to the chain flexibility. (3) The polar proximity factor, F_{Pn} takes into account the reduced hydrophilicity when polar groups are crowded together. This approach is summarized by the following equation:

$\log P = \sum a_n f_n + \sum b_m F_m$

where a, f and n are defined above and b is the number of occurences of factor F of structural type m.

Partition coefficients of peptides

Can the partition coefficient of a peptide be estimated using the group additivity concept? In contrast to the case of small organic compounds or drug molecules, there is little data on partitioning of peptides into octanol. Recently Akamatsu and coworkers (1989) measured the octanol-water partition coefficients of dipeptides and tripeptides. They examined the relationship between log P and the sum of π values of amino acid side chains ($\sum \pi$). There was a linear relationship between log P and $\sum \pi$. The correlation improved with the introduction of structural parameters. However the contribution of the structural parameters depended on the π values used. It seemed that π values obtained from analogues of amino acid side chains might not be the proper values to use for the

prediction of peptide log P. More experimental data are necessary to determine the feasibility of group additivity concept to log P of peptides.

(b) Reverse phase HPLC

Reverse phase HPLC (RPLC) has been widely used in the separation and analysis of various solutes, including proteins or peptides. For separation of peptide mixtures, a linear gradient using water and acetonitrile containing 0.1 % trifluoroacetic acid has been the most frequently used mobile phase (Welling et al, 1987; Guo et al., 1986; Hearn et al., 1988, 1986). The effect of ion-pairing reagents other than trifluoroacetic acid on peptide retention has been described (Guo et al., 1987). RPLC also has been a useful alternative to octanol-water partitioning for the measurement of solute hydrophobicity in QSAR studies.

Retention mechanism of reverse phase HPLC

Solute retention in RPLC is a dynamic equilibrium between the stationary and mobile phases. The solvophobic theory has been a popular model for retention (Braumann, 1986; Yamamoto et al., 1989). In this model, the driving force for retention is the unfavorable interaction of solutes with the water molecules present in the mobile phase. Recently a lattice approach (Dill, 1987; Ying et al., 1989) showed that the driving force for retention is the difference between the binary interaction constants among solutes, mobile phase and stationary phase and the retention mechanism is partitioning rather than adsorption. Dill pointed out the following shortcomings of solvophobic theory: (1) It does not explain the observed linear relationship between the logarithm of the capacity factor and partition coefficient. (2) It is not consistent with the experimental data demonstrating the dependence of retention on the nature of the stationary phase.

Retention in RPLC differs from partitioning into the bulk organic solvents but is similar to partitioning into membranes in that the stationary phase of RPLC is an interfacial phase of highly anisotropic character (Figure 4). One end of the alkyl chain is less ordered than the other where the chain is attached to silica surfaces. The size and shape of the solute as well as its hydrophobicity will influence retention in RPLC as well as partitioning into membranes since the acyl chains in both systems have high surface density and partial order. Braumann (1986) proposed that the stationary phase of RPLC is similar to the polar head group region in biomembranes because of the residual silanol groups, the adsorbed layer of hydrogen-bonding organic solvent and co-extracted water molecules. Therefore, retention in RPLC may be superior to the octanol-water partition coefficient in generating a hydrophobicity parameter related to partitioning into biological membranes.

Hydrophobicity measurement by RPLC

Extensive reviews are available on the subject of using HPLC data for QSAR of organic compounds (Kaibara et al., 1990; Minick et al., 1988) Earlier attempts to mimic the octanol-water partition coefficient by HPLC employed an octanol saturated phase (Mirrless et al., 1976). Alkyl bonded stationary phases such as octadecyl (C18) or octyl (C8) also work satisfactorily for generating retention values that correspond to octanol water partition coefficients. The chromatographic parameter that can be related to the octanol-water partition coefficient P is the capacity factor k'.

$$k' = (t_R - t_0)/t_0$$

where t_R is the retention time of a retained solute and t_0 is the mobile phase hold-up time. The capacity factor is the ratio of the number of solute molecules in the interphase to the number of solute molecules in the mobile phase (Dill et al., 1987). It is related to thermodynamic equilibrium constant K_R via

$k' = K_R\Phi$

where Φ is the phase ratio of the stationary to the mobile phase. Since

$\ln K_R = -\Delta G/RT$,

$\ln k' = -\Delta G/RT + \ln\Phi$.

Comparing the retention data in RPLC with the octanol water partition coefficient has been done using Collander equation:

$\log P_{o/w} = a \log k' + b$

where $P_{o/w}$ is the octanol-water partition coefficient and k' is the capacity factor of RPLC. For small organic molecules methanol-water has been the solvent of choice to obtain k'. A standard curve of log k' versus log $P_{o/w}$ for reference solutes is constructed. Then log $P_{o/w}$ of other compounds can be predicted from their log k'. Although very good correlations are found for some compounds (Brent et al., 1983; Gago et al., 1988), the slope and intercept of this equation are dependent on the mobile phase composition.

To overcome the disadvantage of the isocratic method, log k'_w has been proposed to be a better parameter for describing the hydrophobicity of solutes (Minick et al. 1988, Braumann et al. 1986, Clark et al., 1990). The log k'_w can be determined from the regression of polycratic measurements using the empirical relationship

$\log k'_\phi = \log k'_w - S\Phi_{MeOH}$

where Φ_{MeOH} is the volume fraction of methanol in the eluent and k'_ϕ is the capacity factor measured at that modifier composition. The intercept log k'_w represents the capacity factor under the hypothetical chromatographic condition, 100 % water as mobile phase.

Sometimes a non-linear relationship has been observed and a quadratic equation can describe the relationship;

$\log k' = a_1(\Phi_{MeOH})^2 + a_2(\Phi_{MeOH}) + \log k'_w$

A quadratic equation relating ln k' and Φ is also predicted from lattice theory (Dill, 1987);

$\ln k' = \chi_{AB}\Phi^2 + (\chi_{sB} - \chi_{SA} - \chi_{AB})\Phi + \ln k'_w$.

where χ_{AB} , χ_{SA} and χ_{sB} are the binary interaction constants between the solvents, water (A)

and an organic modifier (B), between the solute and solvent A and between the solute and solvent B, respectively. When $\chi_{AB} \approx 0$, as for the solvent pair of water and methanol, the relationship between ln k' and Φ is linear.

RPLC of peptides

Much effort has been made to predict peptide retention times based on amino acid composition or sequence. The first approach was to examine the relationship between peptide retention time and the sum of Rekker's fragment constants of constitutive amino acids. O'Hare and Nice (1979) first reported that the retention of small peptides could be correlated with the summation of Rekker's fragmental constant.

The second approach was to measure the retention times of a number of peptides of varying composition and find the retention constants for each amino acid by multiple regression analysis. Wehr et al. (1982) reported a linear relationship between the sum of their amino acid fragment constants and the acetonitrile concentration required for elution with a k' of 2 under isocratic condition. This relationship held for peptides with less than 30 residues. Using a linear gradient elution, a linear relationship between peptide retention time and the sum of the amino acid retention constants was observed (Meek et al, 1980). However an exponential relationship between retention time and the sum of retention constants was observed by Sasagawa and coworkers (1982) with a larger number of peptides which ranged from 2 to 80 residues long. To obtain a new amino acid retention coefficient scale, the best fit constants for the system were computed by a non-linear multiple regression analysis (Sasagawa et al., 1982). Their amino acid retention constants predicted peptide retention better than those of Rekker or Meek.

The third approach to define a retention coefficient scale for amino acids was to directly measure retention of a series of synthetic peptides whose composition was systematically changed (Guo et al. 1986a; 1986b). The retention coefficients were used to predict the

peptide retention as follows (Guo et al., 1986a; 1986b). The retention time (τ) of a peptide is the sum of the retention coefficients ($\sum$ Rc), the column dead volume and time correction for the peptide standard (t_s),

$$\tau = \sum R_c + t_o + t_s.$$

When the $\sum R_c$ and τ of standard peptide is known, t_s can be calculated according to the equation; $t_s = \tau - \sum R_c + t_o$. The correlation between predicted and observed retention was 0.98 for 58 peptides (Guo et al., 1986b). However they pointed out that the prediction was not very successful for peptides of 20 residues or greater. The amino acid hydrophobicity scale obtained by this method (Parker et al., 1986) will be discussed later (PGH scale in Table I and V).

Jinno and Tanigawa (1988) looked into the effect of peptide sequence on retention time of small peptides (up to four residues). By introducing a correction factor for the location of each residue, they were able to predict the retention of peptides with about 8 % error when isocratic conditions were employed. However, their retention parameters could not be directly used under other experimental conditions. To apply their retention parameters under other conditions they suggested that the retention of several standard peptides be measured to obtain a correction factor so that the retention parameters for each amino acid could be computed.

In summary, the hydrophobicity of peptides is the major factor determining retention time, at least for peptides of less than 30 residues. However more work is needed before a general prediction method is obtained.

(c) Membrane-water partition coefficient

Membrane organization

The unique property of lipid bilayers as an anisotropic solvent needs to be understood in order to decide which amino acid hydrophobicity scale most appropriately applies to lipid-protein interactions. Lipid bilayers are composed of a polar interface and non polar acyl chains (Figure 4 d). There are various phospholipids found in biological membranes. Depending upon head group structures, they can be positively charged, negatively charged or neutral. The acyl chains vary in length and saturation. The physicochemical properties of phospholipids such as transition temperature, surface density, and miscibility of components vary depending upon the structure of head group and acyl chains. In addition, membrane properties depend upon temperature and the presence of other components such as cholesterol.

The difference between an organic solvent and the acyl chain region of the membrane bilayer is the order of the acyl chains and its anisotropy (Figure 4). The order parameter of acyl chains in the lipid bilayer decreases with the distance from the interface to the midplane of the bilayer. Moreover the bilayer is only 45 Å in thickness so that unlike bulk solvents solute partitioning into membranes will be dramatically influenced by solute bulkiness.

Solute partitioning into membranes

The organization of lipid bilayers has a profound influence on solute partitioning. Extensive studies by Katz and Diamond (1974) showed that partitioning of small molecules into membranes was different from that into bulk solvents. They measured partitioning of nonelectrolytes between dimyristoyl lecithin multilamellar liposomes and water. Partition coefficients between different solvents and water for the same group of solutes were compared according to the Collander equation,

$$\log P_{i,y} = s_{x,y} \log P_{i,x} + r_{x,y}$$

where $P_{i,x}$ or $P_{i,y}$ is the partition coefficient of solute i between solvent x and water or solvent y and water, respectively, while $s_{x,y}$ and $r_{x,y}$ are constants applicable to a

particular choice of solvents x and y. For the solutes used in their study, P between lecithin and water was closer to P between isoamyl alcohol and water than between octanol and water. This seemed to be due to the fact that the group of solutes used were rather polar (eg. small alcohols). These solutes probably do not partition deep into the bilayer but in a region closer to membrane head groups.

They observed a number of differences between partitioning into membranes and bulk solvents: First, membranes discriminated against branched solutes more than a bulk solvent with the same s value. Second the Barclay-Butler slope, $d\Delta S/d\Delta H$, was twice as steep for lecithin as for bulk non polar solvents. For dilute solutions obeying Henry's law, the ΔH and the ΔS of transfer of different solutes from the vapor phase into the same solvent were linearly related. This indicates that change in ΔH is compensated by a change in ΔS. The large slope $d\Delta S/d\Delta H$ for solution into lecithin was attributed in part to the greater immobilization of solutes in bilayers than in bulk solvents. This observation is in accordance with the theoretical approach and experimental results described below.

A lattice model description of solute partitioning into chain molecular interphases such as lipid bilayers, micelles and reverse phase HPLC predicted that there was stable gradients of solute concentration, due to the variation of chain organization with distance from the interface (Marqusee and Dill 1986). This model predicted that an increase of surface density of chains would decrease solute partitioning. The model was confirmed by the observation that partitioning of benzene decreased as the surface density of membranes was increased by temperature, cholesterol or chain length (De Young and Dill, 1988).

Peptide partitioning into membranes

Experimental measurements of peptide partitioning into membranes have been reported by Jacobs and White (1986, 1987, 1989). They measured the partitioning of tripeptides of Ala-X-Ala-OtButyl where X was Gly, Ala, Leu, Phe and Trp into DMPC. However the

peptides did not seem to partition into the acyl chain region. Therefore they defined a so called interfacial free energy of transfer to describe peptide partitioning from an aqueous phase to the polar head group regions. The difficulty involved in determining the location of solutes in the membrane bilayer was also discussed by Katz and Diamond (1974). Since the exact location of the studied solutes might depend on solute properties, they used the term 'average partition coefficient'. Surewicz and Epand (1984) examined the interaction of pentagastrin related pentapeptides with phospholipid vesicles and reported the results as binding instead of partitioning.

When a peptide adopts a secondary structure, intramolecular hydrogen bonds are formed. This reduces the polarity of the peptide bond and partition or incorporation into the membrane bilayers increases (Roseman, 1988; Sneddon et al., 1989).

Jahnig (1983) estimated the free energy of transfer (ΔG) of a hydrophobic α-helix from the sum of the contributions of the hydrophobic effect (ΔG_w), effects of protein immobilization (ΔG_P) and perturbation of lipid order in the membrane (ΔG_L). Hydrogen bond formation that occurs due to a conformational change upon peptide incorporation into membranes did not contribute significantly to the free energy of transfer. The helix was described as a hydrophobic cylinder of radius 5 Å and height 30 Å, the thickness of the hydrophobic core of the bilayer. The hydrophobic effect (ΔG_w) of –35 kcal/mol was obtained by multiplying the surface area of the cylinder by 22 cal/mol $Å^2$ for the free energy per area. Unevenness of the helical surface was accounted for by multiplying the surface area by 1.7. The protein immobilization effect was estimated using a particle in a box approximation. For 0.1 mM lipid dispersion, ΔG_P was 16 kcal/mol. The effect of lipid perturbation by peptide incorporation was estimated to be 2 kcal/mol using a continuum model for lipid order. Therefore the total free energy change, $\Delta G = -17$ kcal/mol, was essentially determined by the hydrophobic effect and the counteracting peptide immobilization effect. This analysis indicates that a peptide would have to be at least 10 residues long before it would partition into the membrane under these conditions.

The result was compared to the experimentally observed data of melittin insertion into DMPC membranes and of cytochrome b_5 binding. The experimental binding energy for melittin was – 9 kcal/mol and for cytochrome b_5 was -11 kcal/mol. Jahnig attributed the difference to the presence of a charged residue in these two peptides. Compared to the elaborate calculation to estimate the free energy of transfer, the statement such as a few kcal/mol difference due to a charged residue seemed to be rather crude. A better estimate of the free energy of transfer of charged residues would help to estimate the hydrophobic effect.

(iii) AMINO ACID HYDROPHOBICITY SCALES

(a) Definition

The idea that the hydrophobicity of individual amino acids is related to their location in a native protein was first suggested by Kauzmann (Kauzmann, 1959). In globular proteins, polar residues are found more often at the surface, where they can interact water, whereas non polar residues tend to be in the interior of proteins avoiding contact with water. An amino acid hydrophobicity scale is the assignment of relative hydrophobicity values to amino acids. Numerous hydrophobicity scales have been derived since the first one was reported by Nozaki and Tanford (Nozaki and Tanford, 1971). They are all based upon the thermodynamics of transfer of side chains from an aqueous phase to a hydrophobic phase. Extensive reviews on the amino acid hydrophobicity scales can be found (Guy, 1985; Rose et al., 1985; Cornette et al., 1987).

(b) Methods to derive amino acid hydrophobicity scales

Various methods to derive amino acid hydrophobicity scales are summarized in Tables, I to IV. The first method is the experimental measurement of solution thermodynamics of amino acids or their analogues (Table I). Distribution of the residues between water and various non-aqueous solvents has been determined. In this approach, the non-aqueous phase is supposed to mimic the interior of the protein or lipid bilayer. However, the non-aqueous phases chosen by different groups, ethanol (Nozaki and Tanford, 1971), octanol (Yunger and Cramer, 1981; Fauchere and Pliska,1983), air-water interface (Bull and Breese,1974), vapor phase (Wolfenden, 1981) and stationary phase of a chromatographic column (Meek et al., 1980; Parker et al., 1986) have different solvent characteristics.

Table I. Measurement of solution thermodynamics to derive amino acid hydrophobicity scales

Nozaki and Tanford, 1971	solubility of free amino acids in ethanol and dioxane
Yunger and Cramer, 1981	octanol-water partition coefficients of free amino acid
Fauchere and Pliska, 1983	octanol-water partition coefficients of N-acetyl amino acid amides
Bull and Breese, 1974	surface tension of aqueous solutions of amino acids
Wolfenden, 1981	hydration potential of amino acid analogues
Meek et al., 1980	retention coefficients of amino acids obtained from the regression studies of peptide retention in reverse phase HPLC
Parker et al., 1986	retention coefficients obtained from the retention time of a series of octapeptides in reverse phase HPLC

The obvious question then is which solvent would best resemble the protein interior or lipid bilayer. The hydrophobicity values assigned from experiments with free amino acids or their analogues do not consider the interactions between neighboring residues or the long range interactions which are possible in peptides or proteins. These considerations have received different treatments by different groups.

The second approach has been empirical calculation from a statistical analysis of proteins of known structure (Table II) (Chothia, 1976; Janin, 1979; Sweet and Eisenberg, 1983; Rose et al., 1985). In this method the frequency a residue is found on the surface or in the interior of the protein is determined from the data base of proteins. The amino acids are rank ordered according to their probability of being in the interior. This method seems to avoid the two problems of (i) a reference solvent, and (ii) long range interactions, raised for the first method because it represents the distribution of residues between the interior and surface of proteins themselves. However, the definition of buried and exposed residues has been somewhat controversial. The relatively small data base used for the calculation also influences the outcome. Moreover, the purpose of the analysis of globular proteins was to predict the structure of globular proteins. It may not necessarily give information for membrane proteins since the membrane is less polar than the protein interior. However the same approach using membrane proteins is even more severely limited due to the small number of membrane proteins available for analysis.

One example is the membrane-buried preference parameters by Argos and coworkers (1982). They used various parameters such as hydration potential (Wolfenden et al., 1981), buried transfer energy (Heijne, 1981), turn preference (Levitt, 1978), residue bulkiness, and polarity (Jones, 1975) to determine the putative membrane spanning segments of several membrane proteins. The algorithm consisted of plotting the amino acid sequence number versus a given parametric value for a particular amino acid and smoothing the curve The plots were smoothed by progressive averaging of seven points.

Table II. Statistical analysis of amino acids distribution in proteins of known structure

Chothia, 1976	Proportion of amino acid residues found 95 % buried in native structure of six proteins
Janin, 1979	Ratio of buried to accessible molar fraction of amino acids measured in 22 proteins
Sweet and Eisenberg, 1983	The average of the amino acids observed to substitute for it in point mutations weighted according to the frequency of substitution.
Rose et al., 1985	Mean fractional area loss for each amino acids in 23 proteins
Argos et al., 1982	Chou-Fasman preference parameters calculated from several membrane proteins
Cornette et al., 1987	Maximization of amphipathic index of power spectra of a set of helices using an eigenvector method

Plots of various parameters were combined and each parameter was weighted appropriately so that the combined plot could predict the structure of bacteriorhodopsin proposed by Engelman and coworkers (1980). The same procedure was performed for several other membrane proteins using the weighting factors determined from bacteriorhodopsin. From these data bases they calculated the membrane-buried preference parameters in an analogous manner to the Chou-Fasman preference parameters for helices. Although the predicted regions in bacteriorhodopsin seemed to agree well with those of Engelman and coworkers, the weighting factors obtained from the analysis of bacteriorhodopsin may not be applicable to other membrane proteins. Moreover, no other evidence of membrane spanning regions were available for most of the membrane proteins used. This approach must be evaluated with more membrane proteins of known structure before its validity will be known.

The third attempt uses the fragment method to calculate the log of the octanol-water partition coefficients of amino acid side chains (Table III).

Table III. Empirical calculation using 'Hansch' type analysis

Abraham and Leo, 1987	Fragment method applied to calculate the log (partition coefficients) of free amino acids and N-acetyl amino acid amides
Roseman, 1988	Fragment method applied to amino acid side chain analogues
Akamatsu et al., 1989	Fragment method applied to amino acid side chain analogues

Hansch π constants (π_X = log P_{RX} - log P_{RH}, where P is the octanol-water partition coefficient) or fragment constants f ($f_X = \pi_X + f_H$) have been widely used for the purpose of

predicting partition coefficients of compounds when experimental values are unavailable. The method is based upon the group-additivity rule and the large number of octanol-water partition coefficients of organic compounds. By proper fragmentation of amino acid side chains and summing the log P of all constituents, octanol-water partition coefficients of side chains can be estimated. The unique problem with amino acids is that when they are situated in peptides, the peptide bond can influence the hydrophobicity of polar side chains. This is known as the polar proximity effect. A decrease of polarity of individual polar groups has been observed in the log P of a number of a organic compounds when the polar groups are close to each other (Leo et al, 1971). Abraham and Leo (Abraham and Leo, 1987) included the polar proximity effect when they calculated the partition coefficients of N-acetyl amino acid amides. However there seems to be a difficulty in predicting the polar proximity effect of ionizable residues. Since a complete set of hydrophobicity values measured by any one of the above procedures does not exist, a combination of the above methods or averages of other scales have been used to obtain a complete set (Kyte and Doolittle, 1982; Eisenberg, 1982; Engelman et al. 1986).

Table IV. Combination of above methods

Kyte and Doolittle, 1982	Combine the Wolfenden scale and the Chothia scale
Eisenberg, 1982	Combine Nozaki and Tanford, Wolfenden, Chothia, Janin and von Heijne
Engelman et al., 1986	Hydrophobic component was calculated from the surface area and the average free energy of transfer (20-25 cal/mol $Å^2$). The hydrophilic component from literature values of the oil-water partition coefficients of polar residues.

The Kyte and Doolittle scale was constructed by averaging the Wolfenden (1981) and Chothia (1976) scales and adjusting the values when necessary. Although this scale is widely used, there is substantial evidence that the value for Trp is incorrect. The hydropathy value for Gly was arbitrarily assigned as the weighted mean of values for all the sequences in their data base because the Wolfenden scale did not include Gly.

The concensus scale of Eisenberg and coworkers (1982) is an average of other scales as shown in Table IV. This scale was designed to mitigate the effects of outlying values in any one scale.

Engelman and coworkers (Engelman et al, 1986) derived a hydrophobicity scale by summing the contribution of hydrophobic and hydrophilic components. The hydrophobic free energy of transfer is proportional to the hydrophobic surface area. According to Reynolds et al. (1974) the value is 20 to 25 cal/mol $Å^2$. Engelman and coworkers took the surface area of amino acid side chains in a helix determined by Lee and Richards (1971) and multiplied by 25 cal/mol $Å^2$ to determine the hydrophobic free energy of transfer. The hydrophilic component was obtained from the oil-water partition coefficient of organic compounds (Davis et al., 1974).

Roseman (1988) critically discussed the shortcomings of Engelman and coworkers' scale. Polar or charged residues seem to pose some problems. First, the polar regions of the residue do not contribute to the hydrophobic free energy change. Therefore the area of nonpolar parts of the molecule should be used to calculate the hydrophobic component. Second, hydrophilic contributions for some residues are not very convincing. For example the hydrophobic component of Trp was – 4.9 and the hydrophilic component was 3.0 and the final hydrophobicity of Trp was – 4.9 + 3.0 = – 1.9 which was close to the value for Ala (– 1.6). For His, the hydrophobic component was – 3.0 while the hydrophilic component was 6.0. Finally, the hydrophilic components obtained from simple organic compounds may be different from those of the polar residues in a peptide.

(c) Comparison among various amino acid hydrophobicity scales

Amino acid hydrophobicity scales

Amino acid hydrophobicity scales which are frequently used or more relevant to the topic of lipid-protein interactions are summarized in Table V. Among the experimentally derived hydrophobicity scales (Table I), Fauchere and Pliska's(F&P), Bull and Breese(B&B), Parker et al.(PGH) and Wolfenden's(WOL) were chosen for comparison. From the second method (Table II), Rose et al. (Rose), Argos et al.(AR) and Cornette et al. (PRILS) were included. Abraham and Leo(A&L) was derived using the fragment method (Table III). Kyte and Doolittle(K&D) and Engelman et al.(ESG) were derived from a combination method (Table IV).

Comparison of hydrophobicity scales

Amino acid hydrophobicity scales can be compared by linear regression analysis of two hydrophobicity scales. The correlation coefficients of such pairwise comparison are summarized in Table VI. The lower half under the diagonal line shows the correlation coefficients calculated from all 20 amino acids. The upper half, shown in italics, contains those obtained with only the non polar amino acids (Gly, Ala, Val, Leu, Ile, Pro, Phe and Trp).

Among the experimentally derived scales (F&P, B&B, PGH and WOL) a reasonable correlation was found between F&P and PGH (R = 0.93) and between PGH and B&B (0.89) when data for 20 amino acids were included in the regression analysis. However when only non polar residues were included, the correlation between F&P and B&B (0.89) improved significantly. The correlation between F&P and PGH and between PGH and B&B also improved

Table V. Amino acid hydrophobicity scales

	F&P	B&B	PGH	WOL	ROSE	AR	A&L	K&D	ESG	PRILS
Gly	0.00	0	-5.7	2.39	0.72	0.62	0.00	-0.4	1.0	-0.40
Ala	0.31	0.200	-2.1	1.94	0.74	1.56	0.32	1.8	1.6	-0.26
Val	1.22	1.560	3.7	1.99	0.86	1.14	1.27	4.2	2.6	1.15
Leu	1.70	2.460	9.2	2.28	0.85	2.93	1.81	3.8	2.8	1.52
Ile	1.80	2.260	8.0	2.15	0.88	1.67	1.81	4.5	3.1	1.10
Pro	0.72	0.980	-2.1	-----	0.64	0.76	0.95	-1.6	-0.2	-0.62
Phe	1.79	2.330	9.2	-0.76	0.88	2.03	1.87	2.8	3.7	1.09
Trp	2.25	2.010	10.0	-5.88	0.85	1.08	1.88	-0.9	1.9	-0.13
Ser	-0.04	0.390	-6.5	-5.06	0.66	0.81	0.01	-0.8	0.6	-0.55
Thr	0.26	0.520	-5.2	-4.88	0.70	0.91	0.33	-0.7	1.2	-0.71
Cys	1.54	0.450	-1.4	-1.24	0.91	1.23	1.05	2.5	2.0	0.83
Met	1.23	1.470	4.2	-1.48	0.85	2.96	1.05	1.9	3.4	1.09
Tyr	0.96	2.240	1.9	-6.11	0.76	0.68	1.20	-1.3	-0.7	0.69
His	0.13	0.120	-2.1	-10.27	0.78	0.29	0.25	-3.2	-3.0	-0.18
Asn	-0.60	-0.80	-7.0	-9.68	0.63	0.27	-0.34	-3.5	-4.8	-0.46
Gln	-0.22	-0.160	-6.0	-9.38	0.62	0.51	-0.91	-3.5	-4.1	-0.83
Asp	-0.77	0.200	-10.0	-10.95	0.62	0.14	-2.55	-3.5	-9.2	-1.30
Glu	-0.64	0.300	-7.8	-10.20	0.62	0.23	-3.60	-3.5	-8.2	-0.73
Arg	-1.01	0.120	-4.2	-19.92	0.64	0.45	-2.51	-4.5	-12.3	0.08
Lys	-0.99	0.350	-5.7	-9.52	0.52	0.15	-1.32	-3.9	-8.8	-1.01

to 0.96 and to 0.92, respectively. This indicates that the relative order of hydrophobicity of non polar residues is not very sensitive to the hydrophobic reference phase whereas the reference phase strongly influences the hydrophobicity value of polar residues.

Correlation among the empirically derived scales was rather poor and remained so when polar residues were excluded from the regression analysis. The ROSE scale showed a reasonable correlation with F&P. Correlation between A&L and the experimentally derived scales F&P, B&B and PGH was improved significantly when only non polar residues were considered in the regression analysis. K&D showed a reasonable correlation

Table VI. Pairwise correlation among different amino acid hydrophobicity scales[a]

	F&P	B&B	PGH	WOL	ROSE	AR	A&L	K&D	ESG	PRILS
F&P	**F&P**	*0.89*	*0.96*	*0.35*	*0.62*	*0.32*	*0.96*	*0.12*	*0.42*	*0.35*
B&B	0.68	**B&B**	*0.92*	*0.10*	*0.62*	*0.31*	*0.97*	*0.27*	*0.52*	*0.60*
PGH	0.93	0.89	**PGH**	*0.25*	*0.74*	*0.44*	*0.94*	*0.23*	*0.59*	*0.51*
WOL	0.69	0.26	0.59	**WOL**	*0.09*	*0.47*	*0.20*	*0.33*	*0.0*	*0.09*
ROSE	0.90	0.47	0.68	0.46	**ROSE**	*0.58*	*0.58*	*0.53*	*0.69*	*0.69*
AR	0.52	0.45	0.57	0.45	0.52	**AR**	*0.31*	*0.44*	*0.47*	*0.58*
A&L	0.79	0.47	0.65	0.54	0.61	0.43	**A&L**	*0.18*	*0.44*	*0.46*
KD	0.81	0.45	0.72	0.89	0.71	0.65	0.58	**K&D**	*0.68*	*0.68*
ESG	0.73	0.35	0.46	0.79	0.62	0.52	0.82	0.72	**ESG**	*0.77*
PRILS	0.77	0.55	0.68	0.33	0.74	0.61	0.47	0.66	0.39	**PRILS**

[a]. The upper half, shown in italics, are the correlation coefficients calculated with only the non polar amino acids (Gly, Ala, Val, Leu, Ile, Pro, Phe and Trp)

with WOL, which is not surprising because it was derived from WOL and the Chothia scale.

The correlation between ESG and A&L was better when 20 amino acids were included in the regression (0.82) than when only non polar residues were included (0.44). It seems that the correction factor for the hydrophilic component of Pro and Trp in ESG is too large. The AR scale, a hydrophobicity scale derived from the membrane protein data base, did not correlate well with other scales probably due to the limited number of membrane proteins in the data base. The correlation of PRILS with any of the other scales in the table was not good.

In the present comparison, the earlier hydrophobicity scales based upon statistical analysis using binary classification of residues into buried and exposed by Chothia and Janin were not included. Earlier correlation studies showed that data from the binary classification approaches correlated poorly with partition energies and better with the hydration energy obtained by Wolfenden (Rose et al., 1985b; Guy et al., 1985). Rose et al. (1985b) explained that the seemingly better correlation between the hydration potential and the empirical calculation by Chothia and Janin is because amphipathic residues favor the aqueous phase in both methods for two unrelated reasons. In the hydration potential measurement of Wolfenden (Wolfenden et al., 1981) there is no compensation for broken hydrogen bonds upon transfer of amphipathic residues to the vapor phase contrary to the organic solvents chosen for the partition experiments such as ethanol, dioxane (Nozaki and Tanford' 1971) or octanol (Yunger and Cramer, 1981; Fauchere and Pliska, 1983). This forces the amphipathic residues to remain in the aqueous phase. In the empirical calculations that uses binary classification, solvent accessibility of amphipathic residues are overestimated due to the stringent criteria for buriedness. Residues were taken to be buried if they had 5 % or less of their potential surface accessible to solvent with the remaining residues classified as accessible. The shortcomings of binary classification were discussed by Guy (1985): (1) Most side chains are neither entirely buried nor entirely exposed to

water. (2) Results depend greatly upon the method used to classify residues as buried or exposed. (3) Binary classification cannot properly represent the situation of amphiphilic side chains concentrating near the water-protein interface.

Attempts to improve the binary classification approach have been made (Prabhakaran and Ponnuswamy,1980; Guy ,1985; Rose et al. ,1985a). Guy (1985) showed that an analysis that divided proteins into layers parallel with their surfaces was more informative than binary classification. Layer analysis by Prabhakaran and Ponnuswamy (1980) correlated better with the hydrophobicity derived from partition energies. Finally the ROSE (Rose et al.,1985a) scale is based upon the average area that each residue buries upon folding and hydrophobicities correlated better with F&P than any other statistical approach (Table III).

Normalized amino acid hydrophobicity scales

An examination of the correlation coefficients among the various scales shows that the hydrophobicity of amino acids depends greatly upon the approaches used to derive the scale. It also shows the importance of the reference phase and the complications arising from polar or charged residues. A comparison of absolute values in the scale is desired in some cases and this can be done only after normalization of original data. Arbitrary normalization has been used to compare the amino acid hydrophobicity scales (Cornette et al., 1987; Parker et al., 1986; Kyte and Doolittle, 1982). In the present study, all scales are presented with the free energy of transfer from water to the non-aqueous phase and the ΔG of Gly is set to 0 for all scales except for values from the WOL scale. The values for other residues are linearly normalized. In Table VII are listed the normalized hydrophobicity values of the scales listed in Table V.

In the F&P scale, the π values of each side chain are determined from experimental measurements of octanol-water partition coefficients of N-acetyl amino acid amides

Table VII. Amino acid hydrophobicity scales based upon Normalized Free Energy of Transfer from water to hydrophobic phase

	F&P	B&B	PGH	WOL	ROSE	AR	A&L	K&D	ESG	PRILS
Gly	0.00	0.00	0.00	-2.39	0.00	0.00	0.00	0.0	0.0	0.0
Ala	-0.42	-0.20	-0.42	-1.94	-0.42	-0.94	-0.32	-2.2	-0.6	-0.14
Val	-1.66	-1.56	-1.06	-1.99	-2.94	-0.52	-1.27	-4.6	-1.6	-1.55
Leu	-2.31	-2.46	-1.66	-2.28	-2.73	-2.31	-1.81	-4.2	-1.8	-1.92
Ile	-2.45	-2.26	-1.53	-2.15	-3.36	-1.05	-1.81	-4.9	-2.1	-1.5
Pro	-0.98	-0.98	-0.42	-----	1.68	-0.19	-0.95	1.2	1.2	0.22
Phe	-2.43	-2.33	-1.66	0.76	-3.36	-1.41	-1.87	-3.2	-2.7	-1.49
Trp	-3.06	-2.01	-1.75	5.88	-2.73	-0.46	-1.88	0.5	-0.9	-0.27
Ser	0.05	-0.39	0.065	5.06	1.26	-0.19	-0.01	0.4	0.4	0.15
Thr	-0.35	-0.52	-0.078	4.88	1.42	-0.29	-0.33	0.3	-0.2	0.31
Cys	-2.09	-0.45	-0.50	1.24	-3.99	-0.61	-1.05	-2.9	-1.0	-1.23
Met	-1.67	1.47	-1.11	1.48	-2.73	-2.34	-1.05	-2.3	-2.4	-1.49
Tyr	-1.31	-2.24	-0.86	6.11	-0.84	-0.06	-1.20	0.9	1.7	-1.09
His	-0.18	-0.12	-0.42	10.27	-1.26	0.33	-0.25	2.8	4.0	-0.22
Asn	0.81	0.80	0.12	9.68	1.89	0.35	0.34	3.1	5.8	0.06
Gln	0.30	0.16	0.01	9.38	2.1	0.11	0.91	3.1	5.1	0.43
Asp	1.05	-0.20	0.45	10.95	2.1	0.48	2.55	3.1	10.2	0.90
Glu	0.87	-0.30	0.21	10.20	2.1	0.39	3.60	3.1	9.2	0.33
Arg	1.37	-0.12	-0.19	19.92	1.68	0.17	2.51	4.1	13.3	-0.48
Lys	1.35	-0.35	-0.02	9.52	4.2	0.47	-1.32	3.5	9.8	0.61

whereas the A&L scale is calculated using fragment method. The values in these scales have been converted to the free energy of transfer term by multiplying by – 1.36 ($\Delta G = -RT\ 2.3 \log P = -1.36\ \pi$). The B&B, WOL and ESG scales show the free energy of transfer from water to the air-water interface, vapor phase and oil, respectively. In Table VII, the sign of the B&B scale has been changed so that the hydrophobicity represents the free energy of transfer from water to the air-water interface. The WOL scale assigns a very unusual hydrophobicity value to the Gly residue whereas the hydrophobicity values of Leu and Ile are comparable to those in F&P. Therefore, in Table VII, only the sign has been changed since any attempt at normalization would have resulted in significant discrepancy with the other scales. The PGH scale was linearly normalized by assigning 0 to Gly and -0.42 to Ala which are the hydrophobicity values for those residues in F&P. The same procedure was followed to normalize ROSE. For PRILS, 0.4 was added to all values so that the hydrophobicity of Gly was 0.

Comparing the normalized values of F&P and B&B shows that the values for non polar residues are consistent between the two scales even though one is the free energy of transfer into octanol and the other is to the air-water interface. For the uncharged polar residues, a significant difference is found only for Cys and Tyr. For the charged residues, the values on the B&B scale are negative which might indicate that the charged residues orient at the air-water interface.

For charged groups such as Arg, Asp and Glu, a significant discrepancy was found between F&P and A&L. Whether the source of the discrepancy is the experimental value (F&P) or the computation (A&L) is not readily apparent. However it indicates that experimental data should be interpreted carefully because complication arising from the experimental system might be overlooked (Abraham and Leo, 1987; Roseman, 1988). The fragment method used by Abraham and Leo is based on the fragment constants given to certain structural units or functional groups. These are derived from experimental measurements of octanol water partition coefficients for thousands of organic compounds. Deviations from the additivity assumption due to structural features such as chain branching

or proximity of polar groups is taken into account in the calculation. Therefore any discrepancy between calculated and experimental data can be attributed either to the complications of the experimental system or to the difficulty in fine-tuning the group additivity relationship. Roseman also used the fragment method to calculate the hydrophobicity of amino acids and noticed a discrepancy with Fauchere and Pliska's data for polar residues. He suggested that the discrepancy is due to deviations from the additivity rule in the model compounds (N-acetylamino acid amide). The deviation is caused by the polar proximity or self-solvation effects.

A number of other unexpected findings can be seen in Table VII. For instance, the hydrophobicity values for Arg and Lys in the normalized PGH scale have a negative sign which is very unusual. Asp and Glu also have a very low hydrophilicity. The differences between Ala and other hydrophobic residues such as Leu, Phe, Trp are not as large as those in F&P or in B&B. This difference might be due to the model compounds used; octapeptides versus N-acetyl amino acids or free amino acids. It could also be due to the difference in the structure of the reference phase. As noted above, RPLC is a more organized structure than the bulk solvents therefore retention time in reverse phase reflects both the hydrophobicity and the bulkiness of the model compounds.

The WOL scale has comparable hydrophobicity values for non polar residues to those of F&P. However the free energies of transfer of polar and charged residues are much more positive which can be explained by the difference in the reference phase; octanol versus vapor phase as discussed above.

The most striking difference between ROSE and experimentally derived scales is the sign of Pro. In ROSE, Pro is as hydrophilic as Arg whereas it is hydrophobic in F&P, B&B and PGH. K&D also assigned a positive value to Pro. The K&D scale was derived from the WOL and the Chothia scales, the latter is based upon the proportion of residues 95 % buried in proteins. Pro is localized at turns due to its unique structure and most turns are at the protein surface. Therefore hydrophobicity values based upon the exposure of

residues such as ROSE or K&D will assign a rather hydrophilic value for Pro. For a similar reason charged residues in ROSE are also more positive than in experimentally derived hydrophobicity scales.

The ESG scale was derived by fragmenting the amino acid residues into a hydrophobic component and hydrophilic component. The hydrophobic contribution to transfer was computed from their surface area using a transfer energy of 20 to 25 cal/Å^2 mol. Therefore the magnitude of the value will depend on the surface area used. The hydrophilic component was obtained using the transfer energy from water to oil documented for organic compounds. The method is based upon the same principle as the fragment method as of the A&L scale discussed above. The values for Val, Leu, Ile and Phe are comparable to those in the F&P scale. Based upon the correspondence of values for hydrophobic residues, the surface areas used by Engelman and coworkers seem to be appropriate. Pro, Trp and His are much more hydrophilic in the ESG scale than in other scales. For these residues, the correction for the hydrophilic component appears to be excessive. For polar or charged residues, the direct addition of the free energy of the polar or the charged groups may not give the correct overall hydrophobicity of the molecule due to the polar proximity effect discussed above.

(d) Questions to be answered

The comparison of various amino acid hydrophobicity scales reveals that they differ considerably for certain residues. For example Pro is hydrophilic in some scales and hydrophobic in others. The magnitude of the absolute values also varied, especially for polar and charged residues.

To get better estimates of amino acid hydrophobicity, the following questions need to be answered. The first question is the effect of the model compound. Free amino acids or amino acid analogues have been used for experimental determination of amino acid

hydrophobicity scales (Yunger and Cramer, 1981; Fauchere and Pliska, 1983; Wolfenden, 1981). It is not known whether the hydrophobicity values derived this way represent values for the side chain in a peptide. In other words the validity of the group additivity assumption is not known. The PGH scale is the only experimentally derived hydrophobicity scale using synthetic peptides. However the PGH scale cannot be compared directly to the other scales because PGH used reverse phase HPLC as a hydrophobic phase while the others employed a bulk phase. Thus the validity of the additivity assumption again cannot be examined directly.

The second question is what is the appropriate reference phase. The difference found between PGH and other experimentally derived scales in Table VII can be attributed either to the structural difference in the parent compounds or to the organization of the reference phase or to a combination of both.

The third question is the hydrophobicity values of polar or ionizable residues. As discussed above discrepancy among the published data for these residues makes it difficult to decide which one is a better hydrophobicity estimate for these residues. The role of intramolecular interaction between the peptide bond and polar side chain is not clearly understood. Hydrophobicity values measured using peptides as model compounds as a function of pH will answer some of these questions.

3. ORGANIZATION OF THESIS

There has been a lack of experimental data of partitioning of model peptides into various hydrophobic phases, which is contrary to the case of many organic compounds and small drug molecules. On the other hand, much effort has been devoted to the design of membrane active peptides or to locate the membrane spanning segments of membrane proteins using currently available amino acid hydrophobicity. However, improvements in

the prediction methods can be achieved only when discrepancies among various amino acid hydrophobicity scales are resolved.

The octanol-water partition coefficient has been the most useful parameter in QSAR involving small molecules. Amino acid hydrophobicity scales derived from octanol-water partition coefficients of free amino acids or amino acid analogues are available. However a scale derived from the octanol-water partition coefficients of peptides has not been reported.

In this study, a series of tripeptides Acetyl-Ala-X-Ala-ONH-t-Butyl, where X = Gly, Ala, Phe, Trp, Pro, His, Asp and Glu, were chosen to examine the hydrophobicity of amino acids in a peptide structure. This series covers a broad range of hydrophobicity but secondary structure formation is not expected. For ionizable residues, His, Asp and Glu, the pH dependence of the partition coefficients was measured. The experimental values fill a considerable gap in current hydrophobicity scales and should be quite useful for the prediction of protein-membrane interactions as a function of pH. In addition, retention time of tripeptides in reverse phase HPLC was compared to octanol-water partition coefficients to see the influence of a different hydrophobic phases. Partitioning into phosphatidylcholine membranes was measured for peptides containing Phe and Trp as central residues. Other tripeptides did not show significant partitioning into the membranes.

Synthesis, purification and characterization of the tripeptides studied are described in Chapter 2. Conventional solution phase methods were employed. Chapter 3 describes the partition coefficient measurements of ^{14}C labeled peptides between octanol and water. For ionizable peptides, the partition coefficients were measured as a function of pH. The side chain contribution of each amino acid to the free energy of transfer between octanol and water was calculated and compared with the published data. Partitioning of tripeptides into phosphatidylcholine liposomes was examined. Thermodynamics of octanol-water partitioning of tripeptides are described in Chapter 4. The thermodynamic parameters are

determined from the temperature dependence of tripeptide partitioning between octanol and water. In Chapter 5 the membrane spanning helices of bacteriorhodopsin and bacterial photoreaction center are predicted using various hydrophobicity scales to examine the impact of inappropriate hydrophobicity values on the prediction.

CHAPTER 2. PEPTIDE SYNTHESIS

INTRODUCTION

A series of tripeptides shown in Figure 1 was synthesized using conventional solution phase methods (Bodanszky and Bodanszky, 1984). These peptides were selected to calculate the amino acids side chain contribution to partitioning into various hydrophobic phases. The advantage of the tripeptides is that contribution from secondary structure formation is not existent while their synthesis is relatively easy. Moreover peptides with blocked N and C terminal will not have complications resulting from ionic interactions. The final intermediates were 9-Fluorenylmethyloxycarbonyl (Fmoc) peptides (Fmoc-Ala-X-Ala-ONH-t-Butyl). Besides serving as a blocking group, fluorescence of the Fmoc group could be used for measurement of partitioning.

To obtain the requisite sensitivity for measuring a wide range of partition coefficients, the tripeptides were radiolabeled using ^{14}C acetic anhydride after removal of the Fmoc group .

MATERIALS

N-tert. Butyloxycarbonyl (Boc)-L-Glycine(BocGly), N-Boc-L-Alanine(BocAla), N-α-Fmoc-L-Alanine(FmocAla), N-Boc-L-Proline(BocPro), N-α-Boc-N-imidazole-Benzyl-L-Histidine(BocHis(im-Bzl)) and N-Boc-L-Aspartic acid-β-Benzyl Ester(BocAsp(O-Bzl)) were purchased from Bachem Biochemicals (Torrance, CA). N-Boc-L-Glutamic acid-γ-Benzyl Ester(BocGlu(O-Bzl) was from Peninsula Laboratories (Belmont, CA). N-Boc-L-Phenylalanine(BocPhe), N-α-Boc-N-indole-Formyl-L-Tryptophan(BocTrp(CHO)) and

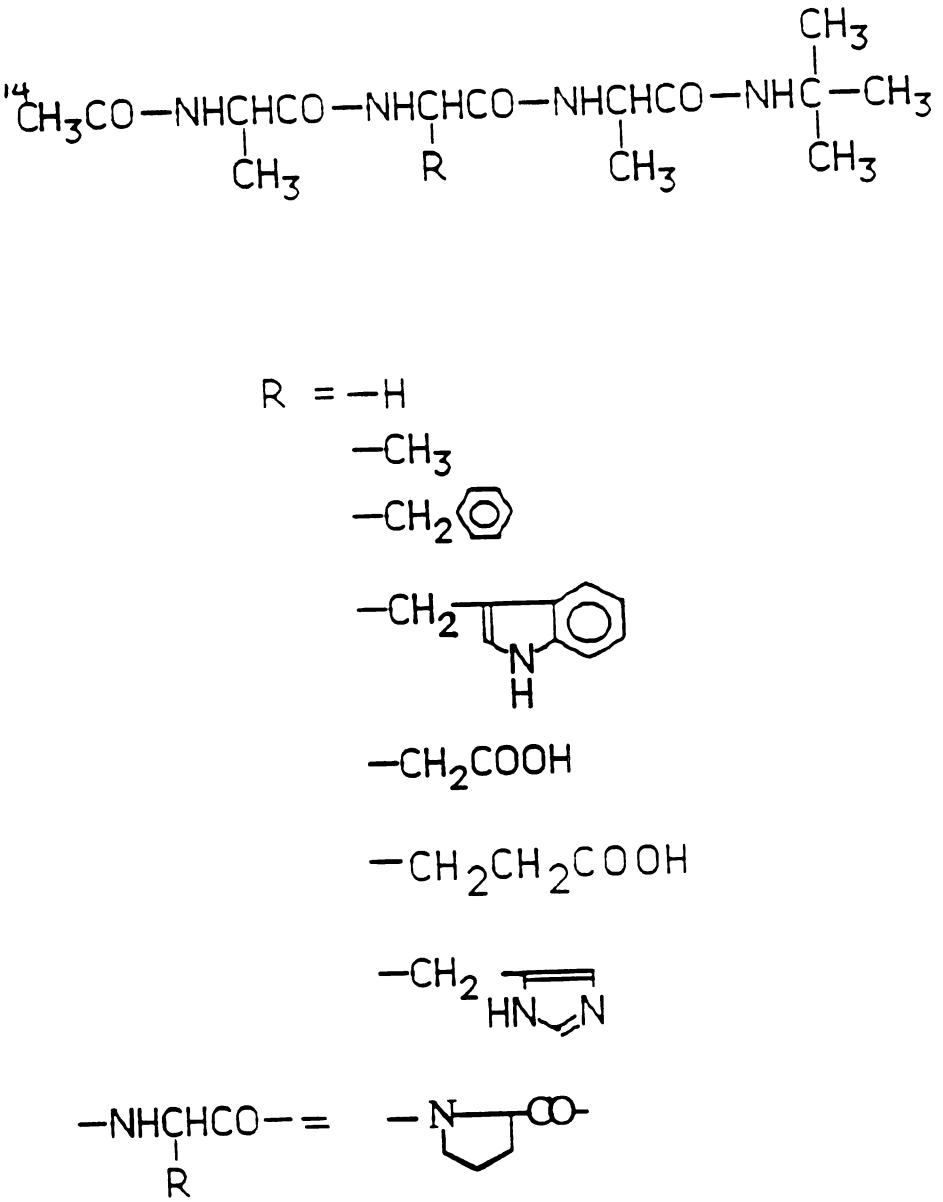


Figure 1. Structure of tripeptides

palladium on charcoal and dicyclohexylcarbodiimide(DCC) were from US Biochemical (Cleveland, Ohio). The blocked amino acids were used without further purification. o-Tolidine dihydrochloride was obtained from Sigma (St. Louis, MO). Chloroform-d($CDCl_3$), 1-hydroxybenzotriazole (HOBT), methyl-d_3 alcohol-d (MeOD), N-methylmorpholine (NMM), pyridine, anhydrous dimethylformamide(DMF) and trifluoroacetic acid(TFA) were from Aldrich (Milwaukee, WI). HPLC grade methanol and acetonitrile and all other ACS grade solvents were from Fisher Scientific (Pittsburgh, PA). All other reagents were analytical grade. Acetic anhydride [1-^{14}C]-$(CH_3CO)_2O$ (10mCi/mmol) in 80 % Benzene was purchased from NEN Research Products (Boston, MA).

METHODS

Conventional solution phase methods were employed for the peptide synthesis. The representative scheme of peptide synthesis is shown in Figure 2 and the representative structures of the intermediates are shown in Figure 3. The mixed anhydride (MA) (Bodanszky & Bodanszky, 1984) or DCC/HOBT method (Bodanszky & Bodanszky, 1984) was used for the coupling reactions. The final intermediates were 9-Fluorenylmethyloxycarbonyl (Fmoc) peptides (Fmoc-Ala-X-Ala-NH-tButyl). Besides serving as a blocking group, the fluorescence of the Fmoc group could also be used for the measurement of partitioning.

Tetrahydrofuran(THF) freshly distilled over $LiAlH_4$ was used as the solvent. The amino acid derivatives or intermediate peptides were dried under high vacuum over P_2O_5 overnight before the reaction. Removal of the Boc group from the intermediates used concentrated HCl/ethyl acetate (1/2) at room temperature (Stahl et al., 1978; Lundt et al., 1978). Side chain blocking groups (benzyl) of Asp, Glu and His were removed by catalytic hydrogenation after completion of the Fmoc tripeptide synthesis (Bodanszky &

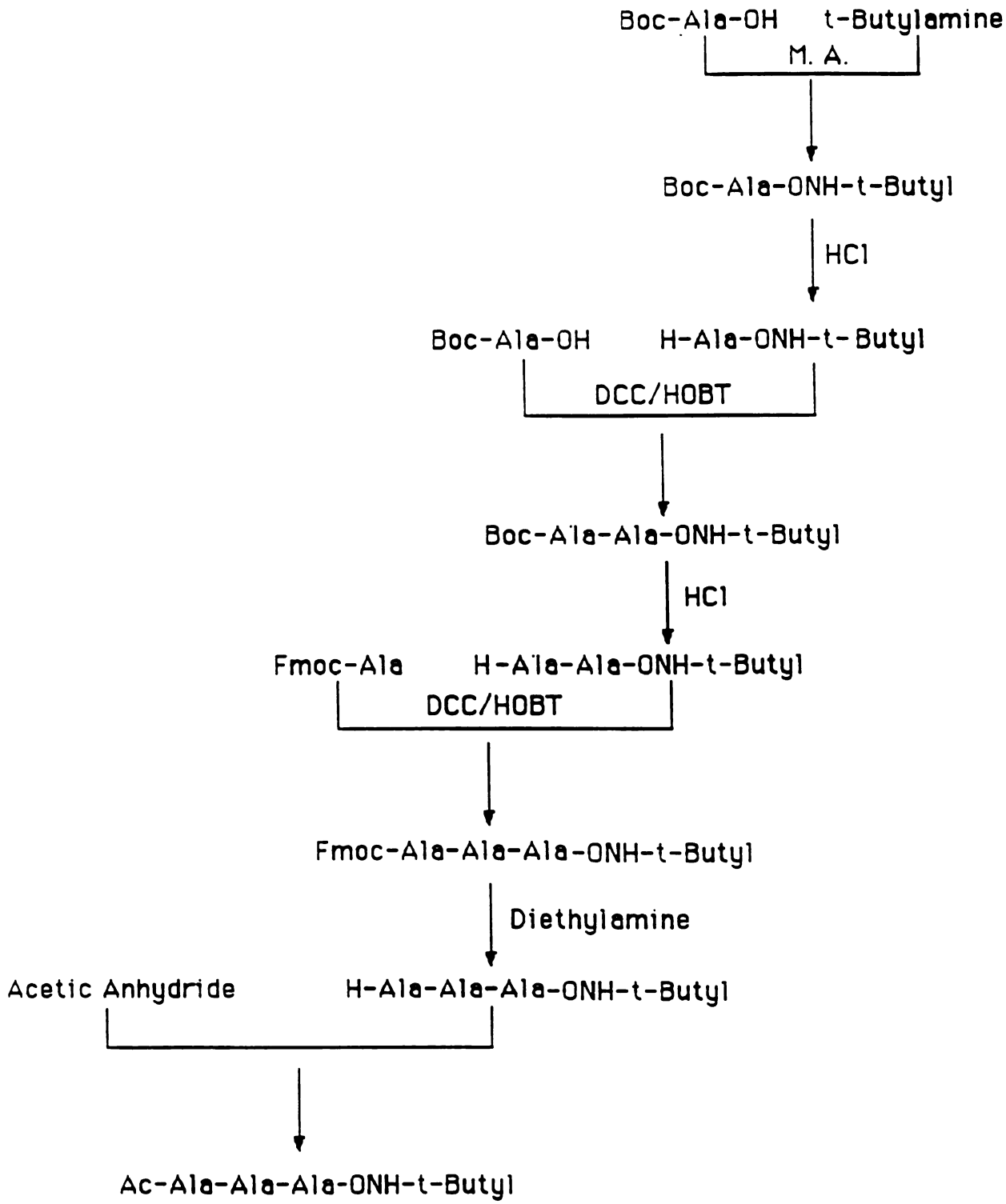
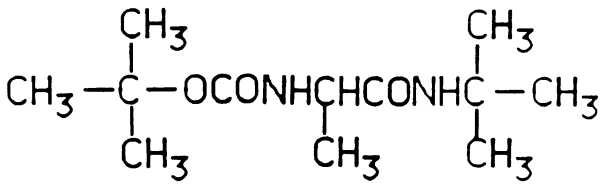


Figure 2. Representative scheme of peptide synthesis

(a)



(b)

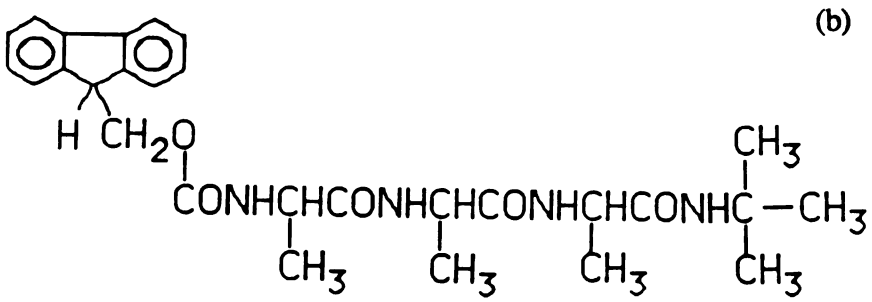


Figure 3. Structure of representative intermediates. (a) BocAlaNHtButyl

(b) FmocAlaAlaAlaNHtButyl

Bodanszky, 1984). The formyl group of Trp was removed by 10 % diethylamine in DMF (Carpino & Han, 1972; Ohno et al., 1972; Previero et al., 1967). TLC on silica gel was used to check the purity of intermediates with ethylacetate/hexane (5/5) or chloroform/methanol (2/1, 3/1, 9/1) as solvent systems. TLC plates were Silica gel 60 (0.2mm, Merck) or MN Silica Gel N-HR/UV_{254} (Macherey-Nagel & Co. Duren). Ninhydrin or chlorine/tolidine spray was used for detection of peptides on the TLC plate. For positive identification of peptides, proton NMR spectra and fast atom bombardment (FAB) mass spectra were obtained. NMR spectra in $CDCl_3$ or MeOD were recorded on a Varian FT-80 NMR Spectrometer using tetramethylsilane as the internal standard or on a GE NMR 300-QE using residual solvent as the reference. Fmoc tripeptides were further purified when necessary on a Dynamax Macro C18 Column (10 mm x 25 cm, 12 μm C18, No 10004) from Rainin Instrument (Emeryville, CA). The peptides were loaded on the column in 250 to 300 μl of chloroform/ methanol (4/1), ethanol or DMF solution The peptide concentrations ranged from 5 mg/ml to 20 mg/ml depending on the solubility. A linear gradient of acetonitrile (0.1 % TFA) and water (0.1 % TFA) with flow rate of 3 ml/min developed over 30 min. Peaks were detected by absorption at 220 nm. The purity and retention times of the various peptides were established on a C18 analytical column (0.46 x 15 cm, 10 μm C18, #27) from Vydac (Herperia, CA) with a solvent flow rate of 1.5 ml/min.

To obtain the requisite sensitivity for measuring a wide range of partition coefficients, the Fmoc was removed and the tripeptides were radiolabeled using ^{14}C acetic anhydride. The Fmoc group was removed by 10 % diethylamine in DMF (Carpino & Han, 1972). The deblocked peptides were dissolved in anhydrous pyridine and reacted with acetic anhydride in the presence of triethylamine at room temperature overnight. Radiolabeled acetylated peptides were purified by preparative TLC (20 x 20 cm, 500 μm, Silica gel GF, Analtech).

I. Fmoc tripeptides

(1) tert. Butyloxycarbonyl-L-alanine tert. Butylamide (BocAlaONHtButyl)

BocAla (1.89 g, 10 mmol) was dissolved in THF (20 ml) in a three neck reaction flask. After the air was displaced with argon, the flask was stoppered with a rubber septum. The flask was immersed in a dry ice/CCl_4 bath (-23 °C). Isobutylchloroformate (1.33 ml, 10 mmol) was slowly added to the reaction flask using a syringe while stirring. After 20 min, t-butylamine (2.73 ml, 20 mmol) was dropped slowly from dropping funnel. The bath was removed and stirring was continued for two more hours. THF was removed under reduced pressure on a rotary evaporator. The residue was dispersed in ethyl acetate and washed with 1 N HCl, 5 % $NaHCO_3$ and 15 % NaCl three times each and dried over Na_2SO_4. Ethyl acetate was removed under reduced pressure on a rotary evaporator. The white residue was further dried over P_2O_5 under high vacuum overnight. The product showed a single spot on TLC with ethyl acetate/hexane (5/5) as solvent (Rf 0.57) and weighed 1.70 g (yield 70 %).

(2) L-Alanine tert. butylamide hydrochloride (HClAlaONHtButyl)

One gram of BocAlaONHtButyl was dissolved in a mixture of ethyl acetate/conc. HCl (4 ml/2 ml). The reaction proceeded for 15 min at room temperature with occasional stirring and TLC (ethyl acetate/hexane(5/5) showed no starting compound as detected by ninhydrin spray. The solvent was removed under reduced pressure on a rotary evaporator. The residue was triturated with diethylether and collected on a filter, washed with diethylether twice and dried over P_2O_5 under high vacuum overnight (yield 87 %).

(3) tert. Butyloxycarbonyl-L-alanyl-L-alanine tert. butylamide (BocAlaAlaONHtButyl)

BocAla (0.735 g, 3.89 mmol) was dissolved in THF (20 ml) in a three neck flask. NMM (0.43 ml, 3.89 mmol) was added. The flask was placed in a dry ice/CCl_4 bath. While stirring, isobutylchloroformate (0.51 ml, 3.89 mmol) was added using a syringe through the rubber septum. After 20 min, a suspension of HClAlaONHtButyl (0.7 g, 3.89 mmol) and NMM (0.45 ml, 3.89 mmol) in 10 ml THF was dropped into the flask. The dropping funnel was rinsed with 5 ml THF. The reaction was stirred for 20 min period and the bath was removed. The further work up was the same as for compound (1) above (yield 87 %).

(4) L-alanyl-L-alanine tert. butylamide hydrochloride (HClAlaAlaONHtButyl)

Removal of Boc group was done as described for compound (2) above (yield not determined).

(5) 9-Fluorenylmethyloxycarbonyl-L-alanyl-L-alanyl-L-alanine tert. Butylamide (FmocAlaAlaAlaONHtButyl)

HClAlaAlaONHtButyl (0.75 g, 3 mmol) was dispersed in THF (50 ml). FmocAla (0.93 g, 3 mmol), HOBT(0.51 g, 3 mmol) and NMM (0.33 ml, 3 mmol) were added. The flask was placed in an ice/water bath. DCC (0.62 g, 3 mmol) was added. The reaction mixture was stirred at 0 °C for an hour and at room temperature overnight. Next day, the reaction flask was placed in ice/water bath and 100 ml of glacial acetic acid was added. After stirring for few minutes, the precipitate was removed by filtration. The solvent was removed under reduced pressure on a rotary evaporator. Ethyl acetate was added to the residue and the precipitate formed was collected on a filter (first crop) and washed with small volume of ethyl acetate several times. The filtrate was washed with 1 N HCl, 5 %

$NaHCO_3$ and 15 % NaCl three times each and the ethyl acetate was dried over Na_2SO_4. After removal of solvent under reduced pressure on a rotary evaporator the residue was combined with the first crop. TLC of the product showed single spot on a silica gel plate using chloroform/methanol (9/1) as solvent system (yield not determined).

(6) tert. Butyloxycarbonyl-L-phenyl-L-alanine tert. butylamide (BocPheAlaONHtButyl)

BocPhe (0.875 g, 3.3 mmol) was dissolved in THF (20 ml) in three neck flask. NMM (0.36 ml, 3.3 mmol) was added. After displacing air with argon, the flask was stoppered and immersed in a dry ice/ CCl_4 bath. Isobutylchloroformate (0.43 ml, 3.3 mmol) was added slowly into the flask using syringe. After 20 min of stirring at -23 °C, a suspension of HClAlaONHtButyl (0.6 g, 3.3 mmol) and NMM (0.36 ml) in THF (10 ml) was dropped from the dropping funnel into the reaction vessel. After 20 min, the bath was removed and stirring was continued for two more hours. Work up procedure was the same as for compound (1) above. An oily residue was obtained and used for the next step without an attempt to crystallize (yield 67%).

(7) tert. Butyloxycarbonyl-L-glycyl-L-alanine tert. butylamide (BocGlyAlaONHtButyl)

HClAlaONHtButyl (1.46 g, 8.1 mmol) was dispersed in THF (30 ml). HOBT (1.38 g, 8.1 mmol), BocGly (1.42 g, 8.1 mmol) NMM (0.89 ml, 8.1 mmol) was added. The reaction vessel was immersed in an ice/water bath. DCC (1.66 g, 8.1 mmol) was added. Stirring was continued for two hours at 0 °C and overnight at room temperature. After addition of 100 ml of glacial acetic acid, the reaction vessel was cooled to 0 °C in an ice/water bath. After stirring for few minutes, the precipitate was removed by filtration. The volume of solvent was reduced by rotary evaporation and the residue was diluted with ethyl acetate and washed with 1 N HCl, 5 % $NaHCO_3$ and 15 % NaCl three times each and

dried over Na_2SO_4. Solvent was removed by rotary evaporation. The residue was dried over P_2O_5 under high vacuum. TLC using ethyl acetate/hexane (5/5) as solvent system showed one major spot (yield 93%).

(8) tert. Butyloxycarbonyl-γ-benzyl -L-glutamyl-L-alanine tert. butylamide (BocGlu(O-Bzl)AlaONHtButyl)

HClAlaONHtButyl (0.95 g, 5.3 mmol), BocGlu(O-Bzl) (1.4 g, 5.3 mmol), NMM (0.58 ml, 5.3 mmol) and HOBT (0.91 g) were dissolved in THF (30 ml). After cooling the reaction flask to 0 °C, DCC (1.09 g, 5.3 mmol) was added. Stirring was continued for one hour at 0 °C and overnight at room temperature. Work up was the same as for compound (7) above (yield, 96 %).

(9) tert. Butyloxycarbonyl-N-formyl-L-tryptophyl-L-alanine tert. butylamide (BocTrp(CHO)AlaONHtButyl).

HClAlaONHtButyl (0.4 g, 2.2 mmol), BocTrp(CHO) (0.66 g, 2.2. mmol), HOBT (0.38 g, 2.2. mmol) and NMM (0.24 ml, 2.2. mmol) were dissolved in THF (30 ml). The reaction flask was cooled in an ice/water bath and DCC (0.45 g,2.2 mmol) was added . Stirring was continued for one hour at 0 °C and overnight at room temperature. The next day, 100 ml of glacial acetic acid was added to the reaction mixture and the flask was cooled to 0 °C. The precipitate was removed by filtration. THF was removed by rotary evaporation. The residue was dissolved in ethyl acetate and washed with 1 N HCl, 5 % $NaHCO_3$ and 15 % NaCl three times each. TLC of the product showed one spot under uv illumination. The ethyl acetate solution was dried over Na_2SO_4 and ethyl acetate was removed by rotary evaporation. The product was dried further under high vacuum over P_2O_5 (yield 94 %).

(10) tert. Butyloxycarbonyl-β-benzyl-L-aspartyl-L-alanine tert. butylamide (BocAsp(O-Bzl)AlaONHtButyl)

HClAlaONHtButyl (1.0 g, 5.6 mmol), BocAsp(O-Bzl) (1.72 g, 5.6 mmol), HOBT (0.96 g, 5.6 mmol) and NMM (0.62 ml, 5.6 mmol) were dissolved in THF (50 ml). After cooling the reaction flask to 0 °C, DCC (1.15 g, 5.6 mmol) was added. The reaction mixture was stirred one hour at 0 °C and overnight at room temperature. The work up was the same as above for compound (9) (yield 82.5 %).

(11) tert. Butyloxycarbonyl-N-imidazole-benzyl-L-histidinyl-L-alanine tert. butylamide (BocHis(im-Bzl)AlaONHtButyl).

HClAlaONHtButyl (1.08 g, 6 mmol), BocHis(im-Bzl) (2.07 g, 6 mmol), HOBT (1.03 g, 6 mmol) NMM (0.66 ml, 6 mmol) were dissolved in THF (30 ml). The reaction mixture was cooled to 0 °C and DCC (1.23 g, 6 mmol) was added. The reaction was stirred for one hour at 0 °C and overnight at room temperature. Next day, the precipitate was removed by filtration and THF was removed by rotary evaporation. The residue was dispersed in ethyl acetate and washed with1 N HCl, 5 % $NaHCO_3$ and 15 % NaCl three times each.. The ethyl acetate solution was dried over Na_2SO_4 and ethyl acetate was removed by rotary evaporation and the product was dried under high vacuum over P_2O_5 (yield 25 %).

(12) tert. Butyloxycarbonyl-L-prolyl-L-alanine tert. butylamide (BocProAlaONHtButyl)

HClAlaONHtButyl (0.394 g, 2.18 mmol) was dispersed in THF (30 ml) and HOBT (0.373 g, 2.18 mmol), BocPro (0.469 g, 2.18 mmol) were added into the flask while

stirring. NMM (0.24 ml, 2.18 mmol) was added and the flask was cooled in an ice/water bath. DCC (0.486 ml, 2.18 mmol) was pipetted into the flask. The reaction mixture was stirred one hour at 0 °C and overnight at room temperature. Work up was the same as above for compound (11) (yield not determined).

(13) 9-Fluorenylmethyloxycarbonyl-L-alanyl-L-glycyl-L-alanine tert. butylamide (FmocAlaGlyAlaONHtButyl)

L-glycyl-L-alanine tert. butylamide hydrochloride (HClGlyAlaONHtButyl) (1.92 g, 8.1 mmol), FmocAla (2.5 g, 8.1 mmol), HOBT (1.38 g, 8.1 mmol) and NMM (0.89 ml, 8.1 mmol) were dissolved in THF (50 ml). The flask was placed in an ice/water bath. DCC (1.66 g, 8.1 mmol) was added and stirring was continued at 0 °C for one and half hours and at room temperature overnight. Next day, after addition of 100 ml of glacial acetic acid, the flask was cooled to 0 °C in an ice/water bath. The precipitate was removed by filtration and the solvent was removed by rotary evaporation. The residue was dispersed in ethyl acetate and washed with1 N HCl, 5 % $NaHCO_3$ and 15 % NaCl three times each and the ethyl acetate solution was dried over Na_2SO_4. The ethyl acetate was removed by rotary evaporation and the residue was dried over P_2O_5 under high vacuum (yield 89 %).

(14) 9-Fluorenylmethyloxycarbonyl-L-alanyl-L-phenyl-L-alanine tert. butylamide (FmocAlaPheAlaONHtButyl)

A solution of FmocAla (1.78 g, 5.73 mmol) in THF (50 ml) was prepared in a three neck flask and NMM (0.63 ml, 5.73 mmol) was added. The flask was stoppered with rubber septum and placed in a dry ice/CCl_4 bath. Isobutylchloroformate (0.74 ml, 5.73 mmol) was added slowly into the flask. After 20 min, a suspension of L-phenyl-L-alanine tert. butylamide hydrochloride (HClPheAlaONHtButyl) (1.88 g, 5.73 mmol) and NMM

(0.63 ml) was slowly dropped into the flask. The reaction mixture was stirred for 10 min, the bath was removed and stirring was continued overnight. The next day THF was removed by rotary evaporation and the residue was dispersed in ethyl acetate and washed with 1 N HCl, 5 % $NaHCO_3$ and 15 % NaCl three times each. Ethyl acetate was removed by rotary evaporation and the residue was washed with a small volume of ethyl acetate several times. The residue gave a single spot on TLC using ethyl acetate/hexane (5/5) as solvent system (yield 89 %).

(15) 9-Fluorenylmethyloxycarbonyl-L-alanyl-N-formyl-L-tryptophyl-L-alanine tert. butylamide (FmocAlaTrp(CHO)AlaONHtButyl)

FmocAla (1.15 g, 3.7 mmol) and NMM (0.41 ml, 3.7 mmol) were dissolved in THF (20 ml) in three neck flask. Air was replaced with argon and the flask was stoppered. After cooling the flask in a dry ice/ CCl_4 bath, isobutylchloroformate (0.48 ml, 3.7 mmol) was added slowly into the flask. After 15 min of stirring, N-formyl-L-tryptophyl-L-alanine tert. butylamide hydrochloride (HClTrp(CHO)AlaONHtButyl) (1.2 g, 3.3 mmol), and NMM (0.41 ml) in THF (10 ml) was dropped into the flask. The dropping funnel was rinsed with additional 10 ml THF. Stirring was continued at -23 °C for 20 min and the bath was removed. Next day, the reaction mixture was diluted with ethyl acetate and washed with 1 N HCl, 5 % $NaHCO_3$ and 15 % NaCl. The precipitate in ethyl acetate was collected by filtration and dried under high vacuum over P_2O_5. TLC of the compound showed a single spot using chloroform/methanol (95.5/0.5) as solvent system (yield 54 %).

(16) 9-Fluorenylmethyloxycarbonyl-L-alanyl-γ-benzyl-L-glutamyl-L-alanine tert. Butylamide (FmocAlaGlu(O-Bzl)AlaONHtButyl)

FmocAla (1.5 g, 5 mmol) and HOBT (0.86 g, 5 mmol) were dissolved in THF (40 ml). And γ-Benzyl-L-glutamyl-L-alanine tert. butylamide hydrochloride (HClGlu(O-Bzl)AlaONHtButyl) (1.64 g, 5 mmol) dispersed in THF (20 ml) and NMM (0.55 ml, 5 mmol) were added. After cooling the reaction vessel to 0 °C, DCC (1.03 g, 5 mmol) was added. Stirring was continued at 0 °C one hour and at room temperature overnight. Next day, 20 ml of THF was added to the reaction mixture because it was too thick to filter. After cooling to 0 °C, the precipitate was filtered and rinsed with chloroform. The solvent was removed by rotavap and the residue was dispersed in ethyl acetate and washed with 1 N HCl, 5 % $NaHCO_3$ and 15 % NaCl three times each. After removal of ethyl acetate by rotary evaporation the residue was repeatedly washed with small volume of ethyl acetate and methanol. The residue gave a single spot on TLC using chloroform/methanol (95.5/0.5) as solvent system (yield 63 %).

(17) 9-Fluorenylmethyloxycarbonyl-L-alanyl-β-benzyl-L-aspartyl -L-alanine tert. butylamide (FmocAlaAsp(O-Bzl)AlaONHtButyl)

β-Benzyl-L-aspartyl-L-alanine tert. butylamide hydrochloride (HClAlaAsp(O-Benzyl)AlaONHtButyl) (1.6 g, 4.33 mmol), FmocAla (1.35 g, 4.33 mmol), HOBT (0.74 g, 4.33 mmol) NMM (0.48 ml) were dissolved in THF. The reaction mixture was cooled to 0 °C and DCC (0.89 g, 4.33 mmol) was added. Stirring was continued one more hour at 0 °C and overnight at room temperature. 100 ml of glacial acetic acid was added and the reaction vessel was immersed in ice/water bath. THF was removed by rotary evaporation and the residue was dispersed in ethyl acetate and washed with 1 N HCl, 5 % $NaHCO_3$ and 15 % NaCl. The white precipitate in ethyl acetate was collected on a filter and washed repeatedly with small volume of ethyl acetate and dried over P_2O_5

under high vacuum. TLC on silica gel using chloroform/methanol (95.5/0.5) gave a single spot detected with chlorine/tolidine (yield 65 %).

(18) 9-Fluorenylmethyloxycarbonyl-L-alanyl-N-imidazole-benzyl-L-histidinyl-L-alanine tert butylamide (FmocAlaHis(im-Bzl)AlaONHtButyl)

N-Imidazole-benzyl-L-histidinyl-L-alanine tert. butylamide hydrochloride (HClHis(im-Bzl)AlaONHtButyl) (0.57 g, 1.4 mmol), FmocAla (0.44 g, 1.4 mmol), HOBT (0.24 g, 1.4 mmol) and NMM (0.16 ml, 1.4 mmol) were dissolved in THF (15 ml) and the reaction mixture was cooled to 0 °C. DCC (0.29 g, 1.4 mmol) was added and stirring was continued for one hour at 0 °C and overnight at room temperature. The precipitate was filtered off and filterate was diluted with ethyl acetate and washed with 1 N HCl, 5 % $NaHCO_3$ and 15 % NaCl. After removal of solvent the yield of crude product was 61 %. TLC of the crude product showed two spots using chloroform/methanol (95/5). Silica gel column chromatography using chloroform/methanol(95/5) gave a 25 % yield of pure compound as determined by TLC. The remainder of fractions were collected and its chloroform/methanol solution (5 mg/1.5 ml) was purified by semipreparative reverse phase HPLC with a linear gradient of 10-80 % acetonitrile/water containing 0.1 % TFA for 35 min.

(19) 9-Fluorenylmethyloxycarbonyl-L-alanyl-L-prolyl-L-alanine tert. butylamide (FmocAlaProAlaONHtButyl)

L-Prolyl-L-alanine tert. butylamide hydrochloride (HClProAlaONHtButyl) (0.49 g, 1.3 mmol) was dispersed in THF (60 ml). HOBT (0.222 g, 1.3 mmol), FmocAla (0.404 g, 1.3 mmol) and NMM (0.143 ml, 1.3 mmol) were added. After cooling the reaction flask to 0 C, DCC (0.29 ml) was pipetted into the flask. The reaction mixture was stirred for

one more hour at 0 °C and then overnight at room temperature. TLC of the mixture using chloroform/methanol (95/1) as solvent system revealed one major product and five minor impurities. After the precipitate was filtered out the solvent was removed by rotary evaporation and the residue was dissolved in a small volume of chloroform/methanol (9/1). The solution was loaded on a preparative TLC plate (20 x 20 cm, 500 mm, Silica gel GF, Analtech) prewashed with chloroform/methanol(9/1). Then it was developed using the same solvent system. The main band was scraped out and extracted with chloroform/methanol(3/1). To remove residual silica gel, the extracted solution was passsed through glass wool plugged disposable pipet and the solvent was removed by rotary evaporation under reduced pressure (yield not determined).

(20) Removal of side chain blocking groups.

Removal of benzyl group from FmocAlaAsp(O-Bzl)AlaONHtButyl, FmocAlaGlu(O-Bzl)AlaONHtButyl and FmocAlaHis(im-Bzl)AlaONHtButyl was done by catalytic hydrogenation. For Asp and Glu peptides, a solution in ethanol (100 mg/100 ml) was prepared in a hydrogenation flask and 10 % palladium on charcoal (15 mg) was added. Vigorous shaking of the flask under 15-20 psi of hydrogen pressure on a Parr hydrogenator was continued overnight. The catalyst was removed by filtration. Semi prep reverse phase HPLC was used to obtain purified deblocked peptides. 250 µl of ethanol solution was loaded and a linear gradient of 20-80 % of acetonitrile in water containing 0.1 % TFA was run for 25 min with 3 ml/min of flow rate. The exact efficiency of hydrogenation reaction was not calculated. However deblocked peptide seemed to be more than 90 % of the reaction mixture judging from the peak areas in the chromatogram. For FmocAlaHis(im-Bzl)AlaONHtButyl, a solution in freshly distilled glacial acetic acid was used for the hydrogenation with 10 % palladium on charcoal. Preparative TLC on silica gel plate (10 x 20 cm, 250 mm, Silica Gel GF, Analtech) using chloroform/methanol/acetic

acid (2/1/0.25) as the developing solvent was done to separate four products. They were found to be AlaHisAlaONHtButyl, AlaHis(im-Bzl)AlaONHtButyl, FmocAlaHisAla and FmocAlaHis(im-Bzl)AlaONHtButyl by mass spectra. Their Rf values under the above purification condition were 0.14, 0.45, 0.80 and 0.95 respectively. Molecular ion peaks found on mass spectra were 353, 443, 575 and 665 respectively. AlaHisAlaONHtButyl obtained this way was used directly for acetylation as described below.

(21) Identification of Fmoc tripeptides

Positive identification of Fmoc tripeptides were done using the mass spectra and NMR spectra (Table I). Fast atom bombardment mass spectra of tripeptides dissolved in methanol were obtained from the Mass Spectrometry Facility, University of California, San Francisco. Molecular ion peaks were observed for all the peptides examined. Mass spectra of His peptide was obtained after removal of blocking group. NMR spectra were obtained in $CDCl_3$ or MeOD and major resonances are summarized in Table I.

II. Acetylation

(1) Removal of Fmoc group

To remove the Fmoc group, Fmoc tripeptides were dissolved in anhydrous DMF and 10 % diethylamine (Carpino & Han, 1972). After two hours at room temperature, the solvent was removed by rotary evaporation at 35 °C. The residue was triturated with diethylether and washed twice with diethylether and dried over P_2O_5 under high vacuum. The deblocking yield was about 90-95 %. For the peptide FmocAlaTrp(CHO)AlaONHtButyl, the mass spectra showed that formyl group was simultaneously removed under this condition.

Table I. Summary of characterization of Fmoc tripeptides

FmocAlaXAlaNHtButyl	Rf[a]	tR[b]	MH+[c]
X=			
Gly	0.25(A)	18.8	495
Ala	0.27(A)	19.2	509
Phe	-	23.1	585
Trp(CHO)	0.36(A)	23.1	652
Pro	0.28(B)	-	535
His(Bzl)	-	19.8	-
Asp(O-Bzl)	0.4(A)	-	643
Glu(O-Bzl)	0.4(A)	24.4	657
His	-	-	665
Asp	-	17.9	553
Glu	0.30(B)	18.2	567

FmocAlaXAlaNHtButyl	chemical shift δ (ppm)[d]			
X=	α	β	γ	others
Gly[e]	3.85			
Ala[f]	4.2(m)	1.39(d)		
Phe[e]		3.05, 3.13		aromatic 7.26
Trp(CHO)[e]		3.3		aromatic
Pro[f]	4.15(m)	1.9-2.16(m)	1.8-2.0(m)	δ3.52(m), 3.66(m)
Asp(O-Bzl)[f]	4.66(t)	2.86(q), 2.92(q)		bzlCH_2,4.6; aromatic,7.26(s)

[a] Rf value obtained by TLC on Silica Gel 60 (Merck); Solvent (A), chloroform/methanol (95.5/0.5); Solvent (B), chloroform/methanol (95/5)

Table I. (continued)

[b] Retention time on a Vydac C18 analytical column (0.46 x 15 cm, 10 μm C18) using a linear gradient of 10-70 % acetonitrile/water containing 0.1 % TFA developed over 30 minute with flow rate of 1.5 ml/min .

[c] Molecular ion peak(MH^+) on fast atom bombardment mass spectra.

[d] Chemical shift (δ) of side chain X relative to the internal standard tetramethylsilane or residual solvent peak. Resonances observed on NMR spectra of all Fmoctripeptides were Fmoc aromatic (7.60,d; 7.74,d; 7.36,t; 7.8,t); Fmoc CH(4.0, t), CH_2(4.4); tert. Butyl CH_3 (d=1.30-1.34, s); Ala $C^{\beta}H_3$ (1.25,d; 1.30,d;exact peak position depended on the central residues), Ala $C^{\alpha}H$ (d=4.1-4.3 quartet or multiplet) and resonances from NH protons observed in $CDCl_3$.

[e] Spectra recorded on Varian FT-80 in CDCl3.

[f] Spectra recorded on GE 300 in MeOD.

(2) Acetylation

The first method used for the acetylation of peptides containing Gly, Ala, Phe, Trp was as follows. After removal of Fmoc group and drying the deblocked peptide over P_2O_5, the peptides were dissolved in dry pyridine and triethylamine (2 eq.), DMAP (0.05 eq.) and acetic anhydride (2 eq.) were added under argon. After reacting overnight at room temperature, pyridine was removed by rotary evaporation. The residue was dispersed in ethyl acetate and washed with 1 N HCl, 5 % $NaHCO_3$ and 15 % NaCl. Using this procedure the % yield of acetylation for the Gly containing peptide was 35 %, for the Phe peptide, 69 % and for the Trp peptide, 64 %. However, the partition coefficient of AcAlaTrpAlaONHtButyl measured by Trp fluorescence was 20 indicating that a significant amount of the peptide was lost during aqueous washing step. Since the simplest synthesis and purification procedure would be desirable for radiolabeling, it was decided that purification would be performed after synthesis directly on preparative TLC without an aqueous phase extraction. It was also found that DMAP, which was added as a catalyst for acetylation, did not enhance the acetylation significantly. Therefore in the radiolabeling procedure, DMAP was left out. Physicochemical characteristics of acetylated tripeptides are summarized in Table II.

(3) Radiolabeling

For radiolabeling of tripeptides, Fmoc peptides were weighed into screw cap tubes. After removal of Fmoc group by 10 % diethylamine in DMF as described above, the residue was triturated with ether (0.5 ml) and centrifuged and ether was removed carefully with a syringe. Ether washing was done three times. Deblocked peptides were dried over

Table II. Properties of acetylated tripeptides

AcAlaXAlaNHtButyl X=	Rf[a]	tR[b]	MH+[c]
Gly	0.66(0.53)	6.2	315
Ala	0.72(0.59)	7.5	329
Phe	0.81(0.74)	24.4	405
Trp	0.79	24	444
Pro	(0.67)	10	355
His	(0.11)	3.8	395
Asp	(0.53)	6.5	373
Glu	-	6.7	387

AcAlaXAlaNHtButyl X=	chemical shift δ (ppm)[d] α	β	γ	others
Gly	3.85			
Ala	4.2-4.5(m)	1.3(d)		
Phe				aromatic 7.18-7.28
Trp	4.5			aromatic 7.0-7.53
His	4.5(t)			aromatic 6.9, 7.8
Asp	4.66(t)	2.86(q), 2.92(q)		bzlCH_2,4.6;aromatic,7.26(s)
Glu	4.2-4.3(m)	1.9-2.2(m)	2.35-2.5(t)	

[a] Rf value obtained by TLC on Silica Gel GF (200 μm, Analtech) using solvent, chloroform/methanol (2/1). Values in the parenthesis are Rf obtained on Silica gel 60 (200 μm, Merck) using solvent, chloroform/methanol(4/1) except Asp and His peptides where chloroform/methanol/acetic acid(150/75/7) was used as solvent system.

[b] Retention time on a Vydac C 18 analytical column (0.46 x 15 cm, 10 μm C18) using a linear gradient of 20-50 % methanol/water containing 0.1 % TFA developed over 30 minute with flow rate of 1.5 ml/min.

Table II (continued)

[c] Molecular ion peak on fast atom bombardment spectra.

[d] Chemical shift (ppm) relative to residual MeOD peak recorded on GE 300. For all peptides following resonances were observed; acetyl CH_3, 2.0 (s); tButyl, 1.3 (s); Ala $C^{\alpha}H$, 4.25(m). Ala $C^{\beta}H_3$ resonance changed depending on the central residues.

Table III. Purification of radiolabeled peptides

Peptides (*AcAlaXAlaNHtButyl)	Gly	Ala	Phe	Trp	Pro	His	Asp	Glu
Solvent system for prep TLC[a]	(A)	(A)	(B)	(B)	(B)	(B)	(C)	(C)
Extraction solvent[b] (chloroform/methanol)	4/1	4/1	4/1	4/1	4/1	4/1	2/1	4/1
% of acetylation[c]	25	27	10	39	28	65	56	51

[a] Preparative TLC plates (20 x 20 cm, 500 μm, Silica Gel GF, Analtech) were used. Solvent system (A), chloroform/methanol (3/1); (B), chloroform/methanol (4/1); (C), chloroform/methanol/acetic acid (150/75/7).

[b] Peptides were extracted from the silica gel scraped off the plate using the solvent system shown.

[c] % yield of acetylation =
(dpm of recovered peptide) / (2 x dpm of ^{14}C-acetic anhydride)

P_2O_5 and weighed. They were kept over P_2O_5 under vacuum until dissolved in dry pyridine for the reaction. Final amount of deblocked peptides in the tubes was 15.8 μmol for Gly, 12.6 μmol for Ala, 8 μmol for Phe, 12.6 μmol for Trp, 12.4 μmol for Asp, 13.7 μmol for Glu and 17 μmol for His containing peptide. 0.5 ml of pyridine was enough to dissolve peptides containing Gly, His and Glu. Others were not completely dissolved in 0.7 or 0.9 ml of pyridine. The tubes were briefly sonicated to give fine dispersion. Benzene was freshly distilled over sodium metal. The procedure described below was performed in a well ventilated hood. [1-^{14}C]-acetic anhydride (1mCi,10 mCi/mmol, 80 % benzene) in a sealed through-joint tube with cap was condensed into the bottom of the tube by immersing the bottom into a dry ice/acetone bath. Then the tube was cut using a file under argon blanket and 450 μl of benzene was added to dilute 10 times and the tube was capped immediately. The tube was allowed to warm up to room temperature in a desicator. All the tubes containing deblocked peptide solutions were placed in a rack under argon blanket. 50 μl aliquot of [1-^{14}C]-acetic anhydride solution (10 μmol) was transfered into the reaction tubes and the tubes were immediately capped. The tubes were shaked gently and allowed to stand at room temperature overnight and stored in a -20 °C freezer until purification.

(4) Purification of radiolabeled peptides

Preparative TLC plates (20 x 20 cm, 500 μm, Silica gel GF, Analtech) were prewashed in the developing solvents summarized in Table III and 1.5 cm from both edges were marked with a glass cutter. The mixture from the acetylation reaction was loaded as a band 2 cm from the bottom of the plate. The plate was developed using an appropriate solvent system shown in the table III below. When the solvent front approached the top of the plate, the TLC plate was removed from the chamber and the premarked edges were cut off. After drying 20 min in the air, the edges were put in chlorine chamber for 10-20 min. Then

Table IV. Summary of peptide synthesis method and yield

Intermediates	Coupling Method[a](%yield)
BocAlaNHtButyl	MA (70)
BocGlyAlaNHtButyl	DCC (94)
BocAlaAlaNHtButyl	MA (87)
BocPheAlaNHtButyl	MA (67)
BocTrp(CHO)AlaNHtButyl	DCC (94)
BocAlaProAlaNHtButyl	DCC (n.d.[b])
BocHis(im-Bzl)AlaNHtButyl	DCC (25)
BocAsp(O-Bzl)AlaNHtButyl	DCC (83)
BocGlu(O-Bzl)AlaNHtButyl	DCC (96)

FmocAlaXAlaNHtButyl X =	Coupling Method[a] (%yield)
Gly	DCC (89)
Ala	DCC (n.d.)
Phe	MA (89)
Trp(CHO)	MA (54)
Pro	DCC (n.d.)
His(im-Bzl)	DCC (n.d.)
Asp(O-Bzl)	DCC (65)
Glu(O-Bzl)	DCC (63)

[a] MA, mixed anhydride method; DCC, DCC/HOBT method

[b] not determined.

they were air dried for10 min and sprayed with tolidine solution. Then both edges were rematched with the TLC plate and the peptide band on this plate was located, removed by scraping and collected in a flask. To quantitate the extent of incorporation of ^{14}C-acetate into the peptide, 1 cm sections from one edge were scraped into scintillation vials and radioactivity was counted in a Beta scintillation counter (Beckman LS-3801). The peptide band was extracted from the silica gel with the chloroform/methanol mixture shown in the Table III. The extracted solution was passed through a glass wool packed disposable pipet to remove residual silica gel. Solvent was removed by rotary evaporation and the residue was dissolved in absolute ethanol. Aliquots were counted and ethanol solutions of all purified peptides were stored in a -20 °C freezer until use.

(5) Identification of radiolabeled peptides

Radiolabeled peptides were identified by TLC in the appropriate solvent system summarized in Tables II. All the radiolabeled peptides showed the same Rf as their corresponding unlabeled acetylated peptides which had been identified with NMR spectra and molecular ion peak on mass spectra (Table II).

RESULTS AND DISCUSSION

Tripeptides with blocked N and C terminal groups (Figure 1) were synthesized by the strategy illustrated in Figure 2. The coupling methods used for each step and reaction yield are summarized in tables IV. BocAlaONHtButyl was prepared using the mixed anhydride method (Bodanszky and Bodanszky, 1984). The product was relatively pure and the yield of the reaction was always 70 % or better. The attempt to use DCC method to make BocAlaONHtButyl from BocAla and tert. butylamine was not successful. The reaction gave an oily residue which did not have a single major product.

Removal of Boc group was performed in conc.HCl/ethyl acetate (1/2) solution; the yield was always about 90 % or better. Deblocked peptide prepared in this way gave a cleaner product in the next coupling reaction than when the peptide was deblocked using trifluoroacetic acid. Trifluoroacetic acid seemed to decompose the t-butylamide bond although this was not pursued further.

The coupling reaction using DCC in the presence of HOBT gave a larger yield of the desired product than when the DCC reaction was run without HOBT. The major byproduct of the coupling reaction using DCC without HOBT was identified as O-acyl-isourea from NMR and mass spectra. The formation of O-acyl-isourea was negligible in DCC/HOBT method. The reaction yield of BocHis(im-Bzl)AlaNHtButyl was particularly low (25 %).

The final intermediates were 9-Fluorenylmethyloxycarbonyl (Fmoc) peptides (FmocAlaXAlaNHtButyl). Characterization of the Fmoc tripeptides is summarized in Table II. Fmoc tripeptides were purified easily by washing with a small volume of solvents such as ethyl acetate or methanol. However, peptides which showed a single spot on TLC sometimes showed a broad impurity peak on HPLC. Later this peak was found to be from the chloroform used to dissolve the peptides. The same peak also appeared when HPLC grade chloroform was used. Fmoc peptides purified by HPLC were used for the final radiolabeling reaction. The peptides gave the expected molecular ion peak on mass spectra and major resonances were found on NMR spectra (Table I).

Hydrogenation of FmocAlaHis(im-Bzl)AlaNHyButyl in glacial acetic acid yielded four products. They were separated by preparative TLC and AlaHisAlaNHtButyl was used directly for the acetylation reaction. Removal of benzyl group of FmocAlaAsp(O-Bzl)AlaNHtButyl and FmocAlaGlu(O-Bzl)AlaNHtButyl used catalytic hydrogenation in ethanol. The disadvantage of the procedure was that a large volume of solution was needed due to the low solubility of the peptides in ethanol. A better solvent would have increased the efficiency of the reaction.

Several different conditions were tried to maximize the acetylation reaction. The yield of acetylation using unlabeled acetyl chloride or acetic anhydride was not significantly different. Acetic anhydride was chosen for the radiolabeling reaction because radiolabeled compound was more readily available. Washing of the reaction mixture with acidic and alkaline aqueous solution was omitted for the acetylation reaction to minimize the loss of the desired product. Preliminary measurement of partition coefficient of AcAlaTrpAlaNHtButyl using fluorescence indicated that the acetylated tripeptides would be lost if washed with aqueous solution. The reaction mixture was purified by preparative TLC. Table II summarizes the properties of the acetylated tripeptides. The composition of TLC solvents and extracting solvents used for the preparative TLC of acetylated tripeptides are summarized in Table III.

CHAPTER 3. OCTANOL/WATER PARTITIONING OF TRIPEPTIDES

INTRODUCTION

The prediction of protein structure or protein-membrane interactions from amino acid sequence depends to a large extent on the hydrophobicity values assigned to the amino acid side chains (Eisenberg, 1984; Engelman, 1986; Cornette, 1987). Although several hydrophobicity scales have been proposed, hydrophobicity values for ionizable residues are inconsistent among studies (Engelman et al., 1986, Fauchere & Pliska, 1983; Abraham & Leo, 1987; Roseman, 1988) and the values at other than neutral pH are rare.

Hydrophobicity is a measure of the relative affinity of a solute for a hydrophobic phase compared to an aqueous phase. The published hydrophobicity scales for amino acids have been compiled in one of three ways: (i) Experimental measurements of free energy of transfer between an aqueous and a non-aqueous phase (Nozaki & Tanford, 1971; Wolfenden et al., 1981; Yunger & Cramer, 1981; Parker et al., 1986). (ii) Statistical analysis of amino acid distribution in proteins of known structure using the criteria that hydrophobic residues are found more often in the interior of globular proteins (Chothia, 1984; Rose et al., 1985) or in contact with the bilayer in the case of membrane proteins (Argos et al., 1982). (iii) Computation based upon a Hansch type analysis where the amino acids are subdivided into chemical fragments and the sum of the fragment constants is used to estimate the hydrophobicity of the side chains(Abraham & Leo, 1987; Roseman, 1988).

The first approach measures the partitioning of amino acids between an aqueous versus a non-aqueous phase. Various non-aqueous phases such as ethanol (Nozaki & Tanford, 1971), vapor phase (Wolfenden et al., 1981), octanol (Fauchere & Pliska, 1983, Yunger & Cramer, 1981) or a C18 reverse phase column (Parker et al., 1986) have been employed in the measurement of the partitioning. Early work by Yunger and Cramer (1981) measured

the octanol-water partition coefficients ($P_{o/w}$) of the 20 amino acids. The hydrophobicity scale based upon these measurements has a number of anomalies due to the charge and potential for hydrogen bonding of the α-amino and α-carboxylate groups. To more accurately account for the influence of a peptide backbone on the hydrophobicity values of the side chains, Fauchere and Pliska (1983) measured the $P_{o/w}$ of N-acetyl amino acid amides. They calculated the contribution (π) of the side chains by subtracting log $P_{o/w}$ of N-acetyl glycine amide from the log $P_{o/w}$ of other derivatives ($\pi = \log P_{o/w(x)} - \log P_{o/w(G)}$). These values are widely used because they are a self-consistent set derived from experimental measurements of all 20 amino acid derivatives. It is not known whether the hydrophobicity contribution of side chains in longer peptides would be the same as the N-acetyl amino acid amides. This should be true if no intramolecular interactions occur in the peptides and hence the additivity rule holds.

Retention time on a reverse phase HPLC is a convenient and rapid alternative method to estimate the hydrophobicity of solutes (Mirrless et al., 1976; Braumann et al., 1986; Minick et al., 1988; Hearn et al., 1988). Parker and coworkers (1986) measured the retention time of a series of peptides up to 8 amino acids on C18 reverse phase HPLC to determine the relative hydrophobicity of amino acid side chains in peptides. There was a good correlation in the relative order between the retention coefficients on the C18 column and Fauchere and Pliska's octanol-water partition coefficients (correlation coefficient = 0.93).

Abraham and Leo (1987) suggested that the fragment method could be used to calculate the $P_{o/w}$ of new compounds or to reassess experimental values of doubtful quality. In applying this method to peptides, (i) the assignment of a fragment value for the peptide bond and the appropriate fragment values for ionizable residues as a function of pH and (ii) the role of proximity effects between peptide bonds and polar residues have yet to be resolved. Moreover there is a discrepancy between the values measured by Fauchere and

Pliska (1983) for Glu and Asp and values computed by the fragment method of Leo and Abraham (1987).

To establish the pH dependent hydrophobicity values for the ionizable residues and to resolve some of the differences between the existing hydrophobicity data, we determined the $P_{o/w}$ and retention times on a C18 column of a series of N and C termini blocked tripeptides (Figure 1 in Chapter 2). Secondary structure formation is not significant in short peptides and blocking of the N and C terminal minimizes complications from ionic interactions between the end groups. The first and third residues are alanine and the central residues are Gly, Ala, Phe, Trp, Pro, His, Asp and Glu. This series of peptides covers a wide range of hydrophobicity values and permits the effect of charge on partitioning behavior to be studied. The side chain contribution ($\pi\chi$) to the free energy of transfer measured using the tripeptides with those obtained with N-acetyl amino acid amides (Fauchere & Pliska, 1983) should reveal complications due to the peptide bond on the additivity assumption.

Although QSAR studies of organic compounds have shown that octanol-water partition coefficients successfully predict the bioactivity of the molecules, few studies on peptides have been reported (Asao et al, 1987). In this study, paritioning of tripeptides into dimyristoylphosphatidylcholine liposomes was measured to see how it is related to the hydrophobicity and the octanol-water partition coefficients of tripeptides.

MATERIALS

1-octanol was HPLC grade from Aldrich and used without further purification. Scintillation cocktail was Ready Value from Beckman. Dimirystoylphosphatidylcholine (DMPC) and palmitoyloleoylphosphatidylcholine (POPC) were obtained from Avanti Polar Lipids Inc. (Birmingham, AL). All other reagents were of analytical grade. Synthesis of peptides was described in the previous chapter.

To ensure the purity of the radiolableled peptides, a series of multiple extractions were performed. First, the partition coefficients between octanol and water($P_{o/w-1}$) of the peptides were measured using glass tubes (16 X 150 mm) containing 5 ml octanol, 5 ml buffer (2mM Tes, pH 7.2, 150 mM NaCl) and aliquots of ethanolic peptide solution. The mixture was vortexed for one minute. The phases were separated by centrifugation and 1 ml of each phase were transfered into glass vials using disposable pipets and the radioactivity was determined in a beta scintillation counter(Beckman LS 3801). The $P_{o/w-1}$ was calculated from the ratio of dpm in the two phases. From the remaining octanol phase, 3 ml was transfered into a clean tube and re-equilibrated with 3 ml of new buffer. After vortexing and centrifugation, radioactivity in 1 ml of each phase was determined and the partition coefficient was calculated ($P_{o/w-2}$). The remaining aqueous phase (3 ml) from the first partitioning was re-equilibrated with fresh octanol and the partition coefficient was measured ($P_{o/w-3}$).

If there were impurities which preferred either the octanol or aqueous phase $P_{o/w-2}$ or $P_{o/w-3}$ would be different from $P_{o/w-1}$. $P_{o/w}$ of AcAlaGlyAlaNHtButyl were all the same indicating this peptide was pure. For AcAlaAspAlaNHtButyl and AcAlaGluAlaNHtButyl, partitioning was performed at pH 2 because at pH 7 the concentration in the octanol phase would be too low to perform a second partitioning. The partition coefficient remained the same after the extraction for these two peptides. Therefore AcAlaGlyAlaNHtButyl, AcAlaAspAlaNHtButyl and AcAlaGluAlaNHtButyl were used without further purification. $P_{o/w-2}$ of AcAlaAlaAlaNHtButyl was significantly larger than $P_{o/w-1}$ (0.33 vs. 0.085) implying the presence of water soluble impurities. The partition coefficient of AcAlaProAlaNHtButyl also changed after extraction.

To determine the % impurity of peptides, reverse phase HPLC was done on a Vydac C18 analytical column (0.46 x 15 cm, 10 μm). Ethanolic solution (10 to 50 μl) of peptide was injected and a linear gradient of 20-50 % methanol in water was run with a flow rate of 1.5 ml/min for 30 minute. Radioactivity was determined with the fractions collected every

minute. Reverse phase HPLC of AcAlaAlaAlaNHtButyl showed that about 30 % of the radioactivity was associated with the solvent peak and the rest of the radioactivity was found at the retention time corresponding to the peptide peak . After repurification of this peptide by preparative TLC, the solvent peak on HPLC chromatogram showed no radioactivity and the three partition coefficients measured by the multiple extraction protocol were the same. AcAlaPheAlaNHtButyl, AcAlaTrpAlaNHtButyl , AcAlaHisAlaNHtButyl and AcAlaProAlaNHtButyl were analyzed by the HPLC procedure and the % impurity was determined to be 2.2, 0.4, 50, and 15 %, respectively. AcAlaTrpAlaNHtButyl was used for the partition experiment without further purification. The partition coefficient of unlabeled AcAlaTrpAlaNHtButyl obtained by the fluorescence measurement was the same as the result of radiolabeled peptide.

Since the impurity was most likely an acetate or a carboxylate, an ethanolic solution of peptides were passed through anion exchange resin(AG1-X8, Formate form, Biorad) to absorb the impurity. Typically, 0.5 g of resin dispersed in ethanol was packed into a disposable polypropylene column (Biorad) (bed volume 1 ml) and rinsed with ethanol. The peptide solution was added to the column and the eluate was collected. The column was washed with an additional 1 ml of ethanol. After the ion exchange chromatography, there was no radioactivity associated with the solvent peak in HPLC. For the partition coefficient measurement described below, AcAlaPheAlaNHtButyl, AcAalProAlaNHtButyl and AcAlaHisAlaNHtButyl were purified by the elution through an ion exchange column.

METHODS

PARTITION COEFFICENTS

Partition coefficients reported here were measured using conventional shake flask method by one day equilibration on a rotator (Sepco Tube Rotator, Scientific Equipment

Products, Baltimore, MD). Samples equilibrated by one or two minutes vortexing or one or two days of rotating gave same result. Typically samples were prepared in triplicate and average was taken for partition coefficient. 5 ml of octanol and 5 ml of buffer (pH 7.3, 2 mM Tes, 150 mM NaCl) were placed in glass screw cap tubes (16 x 150 mm). Ethanolic solution (10 to 30 μl) of peptides was added into tubes using Hamilton syringe to yield a total radioactivity of 1.5×10^4 to 2×10^4 dpm per tube except for the ionized peptides. For the latter case up to 1×10^5 dpm per tube was used. Peptide concentration in aqueous phase ranged 10^{-2}-10^{-4} mM depending on the partition coefficients of peptides. Tubes were securely capped with teflon lined caps and put on a rotator. After one day rotation tubes were centrifuged and 1 or 1.5 ml of both phases were transfered into 20 ml scintillation vials and counted. The partition coefficient ($P_{o/w}$) was calculated from the ratio of DPM in the octanol phase to DPM in the buffer phase. Recovery of peptide from both phase was always in the range of 97 to 107 % indicating no significant peptide adsorption onto the glass. Similar results were obtained using silanized glassware.

PARTITION COEFFICIENTS AS A FUNCTION OF pH

For peptides containing ionizable side chains, partition coefficients at room temperature were measured as a function of pH. Preliminary experiment using an aqueous phase which was titrated with 1 N HCl or NaOH showed a significant pH change after partition measurements. For example, the pH of the aqueous phase (pH 8) dropped to 6 after the partitioning measurement. Therefore aqueous phases were prepared using appropriate buffers: pH 2, titrated with 1 N HCl; pH 3, glycine/HCl; pH 4 and pH 5, acetic acid/sodium acetate; pH 6, 7 and 8, Tes buffers titrated with 1 N HCl or NaOH; pH 9 and pH 10, glycine/NaOH. All buffers contained 150 mM NaCl and the total concentration of buffer species in each buffer ranged between 2-3 mM. Although the buffers differed

among pH studies, the predominant ions were sodium and chloride in all cases to standardize the effect of ions on partitioning (Akamatsu et al., 1989).

To measure the partition coefficient of AcAlaHisAlaNHtButyl, octanol (5.5 ml) and aqueous phase(3 ml) at each pH and 30 µl of peptide solution were equilibrated by rotating at room temperature for one day. After centrifugation, the radioactivity in 5 ml of octanol and 1 ml of aqueous phases was determined. The pH of the remaining aqueous phase was measured using an Orion Ross combination pH electrode connected to Corning pH meter 145. For AcAlaAspAlaNHtButyl and AcAlaGluAlaNHtButyl, octanol (6 ml) and aqueous phase (2 ml) at each pH and 10 or 30 µl of peptide solutions were equilibrated. Octanol (5.5 ml) and buffer (0.5 ml) were transfered into counting vials and the radioactivity determined. The pH of the remaining aqueous phases was measured. Three independent experiments were performed for each peptide.

CALCULATION OF logP USING THE FRAGMENT METHOD

Abraham and Leo used the fragment method to calculate the partition coefficients of the amino acids and their derivatives(Abraham & Leo, 1987). The approach uses fundamental fragment values obtained from partitioning experiments performed on thousands of compounds and is summarized with the following equation:

$$\log P = \sum_{n=1}^{N} a_n f_n + \sum_{m=1}^{M} b_m F_m$$

where a is the number of occurrences of fragment f of structural type n and b is the number of occurrences of factor F of structural type m. The F factors are empirically derived quantities that indicate the increases (+) or decreases (–) in hydrophobicity that

arise from chain or group branching [$F_{cBr(-)}$ or $F_{gBr(-)}$]; bond types [$F_{b(-)}$]; and proximity effects of polar groups with n carbon separation [$F_{Pn(+)}$].

The log P of the tripeptide backbone was calculated using the fragment values (*f*) and other factors (*F*) given in Abraham and Leo (1987). Fragment constant f was defined as $\pi + f_H$ where π was the Hansch π constant ($\pi_x = \log P_{RX} - \log P_{RH}$) and f_H was the fragment constant of hydrogen (0.23). The structures of tripeptides used in this study are shown in Figure 1in Chapter 2. Fragment values for CH_3, CONH and CH are 0.89, – 2.71 and 0.43 respectively. The bond factor F_b corrects for the decrease of log P by 0.12 for each subsequent bond beyond the first C1 to C2 bond. Chain branching decreases log P by 0.13. The polar proximity factor F_{P1} accounts for the decrease of polarity of hydrophilic groups when they are separated by one carbon. In the case of tripeptides, the polarity of the peptide bond -CONH- decreases due to the neighboring peptide bond and F_{P1} is $-0.32(-2.71-2.71) = 1.73$. The log P of the tripeptide backbone can be calculated from the equation $\log P = 6f_{CH3} + 4f_{CONH} + 3f_{CH} + 12F_b + 4F_{cBr} + 3F_{P1} = -0.98$. Therefore the log P of a peptide with side chain R is -0.98 + f_R where f_R is the fragment constant of the side chain R. Fragment constants for side chain (f_R) were those calculated by Abraham and Leo (1987) according to the equation $f_R = \pi + f_H(0.23)$. They were 0.23, 0.55, 2.10, 2.11 and 1.18 for Gly, Ala, Phe, Trp and Pro, respectively. The fragment constants of His, Asp and Glu differed depending on how the polar proximity effects between the peptide bonds and the side chains were calculated. When the polar proximity effect was calculated from the sum of the fragment constants of two peptide bonds (full polar proximity effect), the fragment constant of His was 0.48 and when the average of two peptide bonds was used it was 0.24. The fragment constants of protonated Asp and Glu obtained using full polar proximity effect were 0.63 and 0.22, respectively and for ionized Asp and Glu – 2.32 and – 3.37, respectively.

MEMBRANE-WATER PARTITION COEFFICIENTS

Partitioning of tripeptides into large unilamellar vesicles (LUV) was measured by the equilibrium dialysis method. Chloroform solution of dimyristoylphosphatidylcholine (DMPC) or palmitoyloleoylphosphatidylcholine (POPC) in a round bottom flask was dried by rotary evaporation under reduced pressure until thin lipid film was obtained. The lipid film was dried further under high vacuum overnight to remove residual solvent. Dried lipid film was then hydrated with the buffer (2 mM Tes, 150 mM NaCl, pH 7.2) in 35 °C water bath (DPMC) or at room temperature (POPC) and extruded through 0.1 μm polycarbonate membrane 10 times consecutively. The z average of the liposomes, defined as $\sum N_i M_i^3 / \sum N_i M_i^2$ where N_i and M_i are the number and the mass of the scatters respectively (Billingham, 1977; Martin et al., 1983), was determined with a laser light scattering apparatus (NS-4; Coulter Electronics, Inc., Hialeah, Fla.). They were 140 (± 3) and 150 (± 2) nm for DMPC and POPC LUV, respectively.

A dialysis membrane (Spectropore 3) was placed between the two chambers of a polycarbonate dialysis cell of 1 ml capacity. One chamber was filled with 1 ml of LUV (50 to 100 mM lipid) and the other was filled with 1 ml of peptide solution (10 to 20 x 10^3 DPM) and the chambers were plugged with Nylon screw caps. Control sample had buffer in one chamber and peptide solution in the other chamber. Three dialysis samples were prepared for each peptide. The dialysis samples were placed in an 37 °C incubator and rotated on a rocker overnight. Duplicate or triplicates of aliquots were taken from both chambers and their radioactivity was determined. Lipid concentration was determined by phosphate assay of aliquots of samples from both chambers. Membrane-water partition coefficient ($P_{m/w}$) was determined according to the following equation:

$$P_{m/w} = \frac{[DPM]_{LUV} - [DPM]_{BUF}}{[DPM]_{BUF}} \times \frac{[Water]}{[Lipid]}$$

where $[DPM]_{LUV}$ and $[DPM]_{BUF}$ are the radioactivity of LUV chamber and of the peptide solution chamber respectively and [Water] and [Lipid] are the water and lipid concentration respectively. This equation assumes that the difference of radioactivity between the two chambers corresponded to the radioactivity of peptides associated with lipid. This assumption is true only when the volume occupied by lipid is negligible. If the volume occupied by the lipid phase is considerable, the partition coefficient obtained according to the above equation will be an underestimate of the true partition coefficient.

Partitioning of tripeptides into multilamellar vesicles (MLV) was measured by centrifugation method adapted from Katz and Diamond (1974). MLV of dimyristoylphosphatidylcholine was prepared by hydrating dried lipid film (about 500 μmol lipid) with buffer (5 ml) containing peptide (0.02 μmol) and tritiated water (0.1 μCi/ml) at 35 °C. After overnight stirring in an incubator (37 °C) 1 ml of samples were transfered into preweighed Eppendorf tubes and centrifuged for 20 min in an Eppendorf centrifuge. Supernatants were transfered into preweighed vials and the weight of vials were measured to determine the weight of supernatant. The centrifuge tubes with remaining pellets were weighed and the pellet weight was determined from the difference of the weight of tubes alone and tubes with pellets. After the weights of supernatant and pellet were determined, the radioactivity of vials and pellets were determined using Dual Label DPM program on a liquid scintilation counter (LS 5000 TD) for ^{3}H and ^{14}C. Membrane-water partition coefficient in molal concentration units was determined according to the following equation (Katz and Diamond, 1974):

$$P^{o}_{m/w} = [(\frac{C_{bp}}{C_{bo}} - \frac{C_{tp}}{C_{to}})/(1 - \frac{C_{tp}}{C_{to}})] + f$$

where C_{bp} is DPM of ^{14}C in pellet/gm, C_{tp} is DPM of ^{3}H in pellet/gm, C_{bo} is DPM of ^{14}C in supernatant/gm and C_{to} is DPM of ^{3}H in supernatant/gm. The first term on the right hand side of the equation is the partition coefficient corrected for the trapped water in the pellet assuming the solute concentration in trapped water is the same as in bulk water. The

second term, f, corrects for the non solvent water which is defined as water bound strongly to lipid and is unavailable for dissolving solute. The $P^{\bullet}_{m/w}$ can be converted to the partition coefficient on mole fraction basis ($P_{m/w}$) by multiplying with the ratio of molecular weight of water and lipid.

RESULTS AND DISCUSSION

pH DEPENDENCE OF $P_{o/w}$ OF CHARGED PEPTIDES

The pH dependence of $P_{o/w}$ was examined for AcAlaAspAlaNHtButyl, AcAlaGluAlaNHtButyl and AcAlaHisAlaNHtButyl (Figure 1). In this data $P_{o/w}$ is the apparent partition coefficient since no attempt was made to find the partition coefficient of the uncharged form. AcAlaAspAlaNHtButyl and AcAlaGluAlaNHtButyl are mostly ionized at pH 10 and uncharged at pH 2. AcAlaHisAlaNHtButyl are predominantly ionized at pH 3 and uncharged at pH 9. As expected the $P_{o/w}$ becomes larger as the uncharged fraction increases. At all pH values AcAlaGluAlaNHtButyl has a larger $P_{o/w}$ than the $P_{o/w}$ of AcAlaAspAlaNHtButyl due to the contribution of the additional methylene group in Glu. The $P_{o/w}$ of uncharged and ionized peptides are summarized in Table I.
The difference of log $P_{o/w}$ between the ionized and uncharged forms (Δ log $P_{n/ion}$) for each peptide was calculated and included in Table I. For AcAlaHisAlaNHtButyl, AcAlaAspAlaNHtButyl and AcAlaGluAlaNHtButyl, Δ log $P_{n/ion}$ was 1.57, 2.65 and 2.48, respectively. For aromatic amines, Δ log $P_{n/ion}$ is 3.9 and for aliphatic acids it is about 4.0 and for salicylic acid it is 3.1 (Leo et al., 1971). The Δ log $P_{n/ion}$ of the tripeptides and those of simple organic compounds differ by more than 1 log unit. Therefore the effect of charge on the partition coefficient of tripeptides cannot be predicted from the behavior of simple organic molecules.

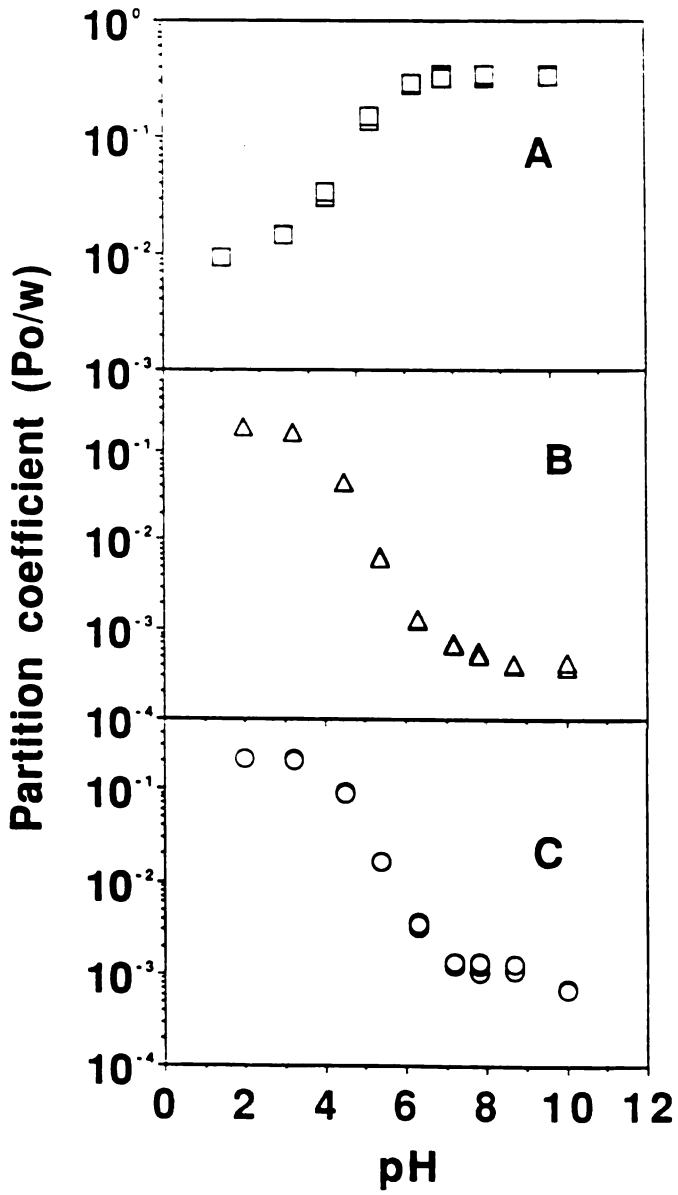


Figure 1. pH dependence of partition coefficients for peptides AcAlaHisAlaNHtButyl (A), AcAlaAspAlaNHtButyl (B), and AcAlaGluAlaNHtButyl(C). The partition coefficient was measured as described in the methods. Each symbol represents the data point from three independent measurements. The symbols overlap.

Table I. pH dependence of partition coefficients of ionizable peptides

AcAlaXAlaNHtButyl X =	P_n[a]	P_{ion}[b]	$\Delta logP_{n/ion}$[c]
His	0.35	9.3×10^{-3}	1.57
Asp	0.18	4×10^{-4}	2.65
Glu	0.21	6.9×10^{-4}	2.48
π_{CH2}[d]	0.067	0.24	

[a] The partition coefficients of peptides at pH values where they are predominantly uncharged; pH 9 for His; pH 2 for Asp and Glu.

[b] The partition coefficients of peptides at pH values where they are predominantly ionized; pH 3 for His; pH 10 for Asp and Glu.

[c] $\Delta logP_{n/ion} = \log P_n - \log P_{ion}$.

[d] π_{CH2} is the difference between partition coefficients of Asp and Glu peptides in log unit. $\pi_{CH2} = \log P_{(AcAlaGluAlaNHtButyl)} - \log P_{(AcAlaAspAlaNHtButyl)}$.

The contribution of one methylene group (π_{CH2}) calculated by subtracting log P of AcAlaAspAlaNHtButyl from log P of AcAlaGluAlaNHtButyl differed depending on the pH (Table I). For the protonated peptides it was 0.067 and for charged peptides it was 0.24. The average of π_{CH2} in hydrocarbons is 0.5 but is reduced when the CH_2 is between two very polar groups or the methylene chain folds back upon itself (Leo et al., 1971). The π_{CH2} computed using Asp and Glu peptides is lower than that computed using a hydrocarbon chain. This is because difference of log P between the two peptides is determined not only by one additional methylene in Glu but also by the difference in the polarity of the peptide bond and the carboxylate between AcAlaGluAlaNHtButyl and AcAlaAspAlaNHtButyl. The polar proximity effect between the peptide bond and the carboxylate in Glu is less than in Asp because of the longer distance between the polar groups in Glu. Therefore the peptide bond and carboxylate group in Glu are more polar than those in Asp. As a result, π_{CH2} is less than the average values found in aliphatic compounds.

The difference of π_{CH2} between the charged and the protonated forms is again due to the polar proximity effect. The effect propagates further in charged groups than uncharged polar groups. Therefore the polarity of these groups in Glu and Asp will be more similar in the charged form and the π_{CH2} (0.24) in charged form will appear to be greater than the π_{CH2} (0.067) in the protonated form.

Ionizable peptides partition into the octanol phase in both the uncharged and ionized forms. The $P_{o/w}$ of AcAlaGluAlaNHtButyl measured at pH 10 was orders of magnitude greater than $P_{o/w}$ calculated assuming only the uncharged form can partition into octanol. $P_{o/w}$ can be expressed as:

$$P_{o/w} = \frac{[HA]_o + [A^-]_o}{[HA]_w + [A^-]_w} .$$

where [HA] is the protonated form and [A^-] is the ionized form of the peptide. If only HA can partition into the octanol phase, $P_{o/w}$ can be written as follows:

$$P_{o/w} = \frac{[HA]_o}{[HA]_w + [A^-]_w}$$

At pH 2.0 the Glu peptide (pKa = 4.25) is more than 99.7 % protonated and the ratio $[HA]_o/[HA]_w$ is approximately the same as $P_{o/w}$. Therefore $[HA]_o/[HA]_w = 0.21$ and $[HA]_o = 0.21\ [HA]_w$. At pH 10, $[A^-]_w$ can be calculated using Henderson-Hasselbach equation and $[A^-]_w = 56234\ [HA]_w$.

Therefore $P_{o/w}$ at pH 10 will be

$$P_{o/w} = \frac{0.21[HA]_o}{[HA]_w + 56234[HA]_w}$$

$$= 3.73 \times 10^{-6}$$

The measured value at pH 10 was 6.9×10^{-4} , i.e., two orders of magnitude greater than the calculated value. This indicates that partitioning of the ionized peptides must be the major contribution to the apparent partition coefficient at this pH.

MEASURED VS. CALCULATED PARTITION COEFFICIENTS OF TRIPEPTIDES

Values of log P measured experimentally and computed using the fragment method of Abraham and Leo (1987) are presented in Table II. In most cases the computed values agree quite well with our experimentally measured log P of peptides for Gly, Ala, Phe and Trp peptides. The log P of tripeptides seems to be mainly determined by the constituent groupings according to the group additivity rule. A large difference was found between the experimental and calculated log P of AcAlaProAlaNHtButyl. When the fragment constant of hydrogen, f_H (0.23) was added to account for the difference in the backbone structure the difference was still larger than other non polar peptides. This indicates that the

fragment method predicts a log P for AcAlaProAlaNHtButyl that is inconsistent with our experimental measurements.

For peptides containing ionizable residues, His, Asp and Glu, the calculation using the full polar proximity effect gave a better agreement between the experimental and calculated log P of the tripeptides. For AcAlaGluAlaNHtButyl, the experimentally obtained logP at pH 10 is significantly greater than the calculated logP. The fragment value for the side chain of Glu calculated by Abraham and Leo seems to be too low. According to their calculation (Abraham & Leo, 1987), Glu is more hydrophilic than Asp. However our and others (Fauchere & Pliska, 1983; Wolfenden, 1981; Parker et al, 1986) experimental evidence indicates that Glu is more hydrophobic than Asp due to the additional methylene . The difficulty in predicting log P of polar residues seems to be in assigning a polar proximity effect. Abraham and Leo added only a 10 % proximity effect to obtain the log P of the Glu residue because there are three carbons between the peptide bond and the carboxylate. For Asp residue, however, where two carbons exist between peptide bond and carboxylate, a 26 % proximity effect was added to obtain the log P. As a result Glu is computed to be less hydrophobic than Asp which is inconsistent with the experimental data. This points out a current limitation of such calculations as applied to peptides.

Table II. Comparison of experimental and calculated log P of tripeptides

AcAlaXAlaNHtButyl					
X =	Gly	Ala	Phe	Trp	Pro
log P_{expt}	– 0.60	– 0.51	1.01	1.25	– 0.39
log P_{calc}	– 0.75	– 0.43	1.12	1.13	0.20 (– 0.03[b])
Δ log P[a]	0.15	– 0.08	– 0.11	0.12	– 0.59 (– 0.36[b])

X =	His (pH 7)	Asp (pH 2)	Asp (pH 10)	Glu (pH 2)	Glu (pH 10)
log P_{expt}	– 0.48	– 0.74	– 3.40	– 0.67	– 3.15
log P_{calc}	– 0.74 (– 0.50)	– 0.98 (– 0.35)	– 3.93 (– 3.3)	– 1.0(– 0.76)	– 4.59 (– 4.35)
Δ log P[a]	0.26 (0.02)	-0.24 (0.23)	0.57 (– 0.10)	0.33 (0.09)	1.44 (1.2)

[a] log P = log P_{expt} -log P_{calc}, where P_{expt} is the measured octanol-water partition coefficient of the tripeptides and P_{calc} is the partition coefficient calculated using the fragment method as described in the method. The values in the parenthesis are those calculated using full polar proximity effect for charged residues (Abraham & Leo, 1987).

[b] log P of Pro peptide was calculated from the equation log P = -0.98 + f_R - f_H(0.23) to account for the difference in the backbone structure.

MEMBRANE-WATER PARTITION COEFFICIENTS

Partitioning of tripeptides into liposomes was not detectable except for AcAlaPheAlaNHtButyl and AcAlaTrpAlaNHtButyl. The $P_{m/w}$ of AcAlaPheAlaNHtButyl and AcAlaTrpAlaNHtButyl into POPC LUV were 30 (± 2) and 396 (+24), respectively. The $P_{m/w}$ of AcAlaTrpAlaNHtButyl into DMPC LUV was 341 (± 2). The $P_{m/w}$ into MLV were 86 (± 4) and 482 (± 114), respectively when the value for f factor was assumed to be 0.3. Without the correction factor f, they were 75 and 470 for Phe and Trp peptides, respectively. The difference between the results of LUV and MLV seems to be due to the difference in experimental methods. For equilibrium dialysis of LUV, the equation used to calculate the partition coefficient assumes that the peptide concentration inside the liposomes is the same as in bulk water. If during the time scale of a day peptides do not readily partition into the inner aqueous phase the measured partition coefficient will be an underestimate. On the other hand MLV were prepared by hydrating with buffer containing peptides and hence true equilibrium would be reached fast. One unknown of this experiment was the fraction of non solvent water. However for Phe and Trp peptides the f factor made less than 10 % of difference. Therefore the result with the MLV seems to be closer to the true partition coefficient.

Diamond and Katz (1974) examined the relationship between octanol-water partition coefficient ($K_{o/w}$) and membrane-water partition coefficient ($K_{m/w}$) of small non electrolytes. The Collander equation for the two partition coefficients was as follows:

$\log K_{m/w} = 0.87 \log K_{o/w} - 0.13$

where $K_{m/w}$ and $K_{o/w}$ are octanol water partition coefficient and membrane water partition coefficient on a molal basis. Assuming that tripeptides partition into DMPC membranes in the same manner as the solutes used by Katz and Diamond, membrane-water partition coefficients of tripeptides can be predicted from their octanol-water partition coefficients using the Collander equation. The membrane-water partition coefficients of tripeptides

predicted in this manner on a mole fraction basis are 9.8, 12, 269, 403, 94 and 13 for Gly, Ala, Phe, Trp, Pro and His peptides respectively. The predicted value of Trp peptide is close to the measured value and for Phe peptide it was about three fold less. It may be that these two peptides interact with membranes in a different manner. According to the prediction, it is not surprising that partitioning of Gly, Ala and His is undetectable. With 200 mM of lipid concentration the radioactivity of lipid chamber and of the buffer chamber would be 10438 vs. 10000 for Ala peptide. The difference is in the range of experimental error. Therefore with the lower lipid concentration used in the present experiment the difference would be even smaller and partitioning would be undetectable. The Pro peptide did not show significant partitioning despite the predicted value of 94. It is not clear whether the $P_{m/w}$ of AcAlaProAlaNHtButyl was not detectable simply due to the low resolution (1170 DPM vs. 1000 DPM with 100 mM lipid concentration) or there are other factors influencing the partitioning of Pro peptide and the peptide actually partitions into membrane less than Collander equation predicts. It may also be that the Pro peptide, like the Phe peptide, partitions into membranes three fold less than the Collander equation predicts and cannot be detected by the methods used in the present study.

Comparison between the measured and predicted membrane-water partition coefficients of tripeptides shows that the octanol-water partition coefficient can give rough estimation of the membrane-water partition coefficients. Therefore it will be beneficial for the purpose of peptide designing if one can measure or predict octanol-water partition coefficients of peptides more precisely to get desired hydrophobicity.

HYDROPHOBICITY OF AMINO ACID SIDE CHAINS

The log $P_{o/w}$ of tripeptides can be used to derive the amino acid side chain hydrophobicity in an analogous manner to Fauchere and Pliska (1983). The values of log Po/w are presented in Table II and the amino acid side chain contributions, π and ΔGx are

summarized in Table III. The hydrophobicity value π of the central residue in AcAlaXAlaNHtButyl is determined by subtracting log $P_{o/w}$ of AcAlaXAlaNHtButyl from log $P_{o/w}$ of the peptide; π = log $P_{o/w(AcAlaXAlaNHtButyl)}$ - log $P_{o/w(AcAlaGlyAlaNHtButyl)}$. A more general term to express the side chain hydrophobicity is the contribution of residue X (ΔG_x) to the free energy of transfer of the peptides and can be calculated by the equation, $\Delta G_x = -RT\ 2.3\ \pi$ or $\Delta G_x = \Delta G_{(AcAlaXAlaNHtButyl)} - \Delta G_{(AcAlaGlyAlaNHtButyl)}$, where the ΔG terms in the right hand side are the free energies of transfer of tripeptides from water to octanol.

The free energy of transfer (ΔG) of the various tripeptides from aqueous solution to octanol was calculated using the equation, $\Delta G = -2.3\ RT \log P$, where P is the partition coefficient calculated using the ratio of mole fraction of peptide in the octanol and aqueous phases. $P_{o/w}$, the ratio of molar concentration in octanol and buffer phases can be converted to P by multiplication of the molar volume ratio of the two solvents so that $P = P_{o/w}\ V_{oct}/V_{water} = 8.748 \times P_{o/w}$ assuming the molar volume ratio of the two solvents remains the same over the temperature range studied.

COMPARISON WITH OTHER HYDROPHOBICITY DATA

The hydrophobicity values of the residues examined in this study and those selected from various published data are presented in Table III. Hydrophobicity data shown in the first section of Table III are the original values reported as either π (Fauchere & Pliska, 1983; Abraham & Leo, 1987; Roseman, 1988;Akamatsu et al, 1989) or retention coefficients (Parker et al., 1986; Guo et al., 1986) or ΔG_x (Engelman et al., 1986; Kyte & Doolittle, 1982). The second section presents the data converted to the same unit, ΔG_x.

Table III. Comparison of hydrophobicity parameters from tripeptides with published data.

Peptides (AcAlaXAlaNHtButyl)	Gly	Ala	Phe	Trp	Pro	His	Asp	Glu	R1[s]	R2[t]
π(tripeptides)[a]	0	0.09	1.61	1.86	0.44	0.12	-2.57	-2.29	1.00	1.00
π(F&P)[b]	0	0.31	1.79	2.25	0.72	0.13	-0.77	-0.64	0.86	0.96
HPLC[c]	0	0.69	9.58	9.37	2	-1.26	0.16	0.27	0.98	0.95
PGH(pH7)[d]	0.0	2.2	9.0	9.5	2.2	2.2	-2.6	-1.3	0.83	0.97
PGH(pH2)[e]	-0.2	2.0	8.1	8.8	2.0	-2.1	0.2	1.1	0.98	0.98
K&D (Kcal/mol)[f]	-0.4	1.8	2.8	-0.9	-1.6	-3.2	-3.5	-3.5	0.45	0.45
ESG (Kcal/mol)[g]	-1.0	-1.6	-3.7	-1.9	0.2	3.0	9.2	8.2	0.87	0.39
π(A&L)[h]	0	0.32	1.87	1.88	0.95	0.01(0.25)	-3.18(-2.55)	-3.84(-3.60)	0.95	0.83
π(Roseman)[i]	0	0.39	2.27	2.13	-	-0.64	-3.81	-2.91	0.97	0.89
π(Akamatsu)[j]	0	0.32	1.95	1.92	0.86	-	-	-	0.92	-
ΔGx(Kcal/mol)[k]	0	-0.13	-2.19	-2.52	-0.29	-0.16	3.50	3.12		
ΔGx(Kcal/mol)(F&P)[l]	0	-0.42	-2.43	-3.06	-0.98	-0.18	1.05	0.87		
PGH(Kcal/mol)[m]	0	-0.42	-1.73	-1.81	-0.42	-0.42	0.50	0.25		
K&D(Kcal/mol)[n]	0	-2.2	-3.2	0.5	1.2	2.8	3.1	3.1		
ESG(Kcal/mol)[o]	0	-0.6	-2.7	-0.9	1.2	4.0	10.2	9.2		
A&L(Kcal/mol)[p]	0	-0.43	-2.54	-2.56	-1.29	0.46	4.32	5.22		
Roseman(Kcal/mol)[q]	0	-0.49	-3.09	-2.90	-	0.87	5.18	3.96		
Akamatsu(Kcal/mol)[r]	0	-0.43	2.65	2.61	1.17	-	-	-		

Table III (continued)

a. The hydrophobicity data obtained in this study from the octanol-water partition coefficients of tripeptides at pH 7.2; $\pi = \log P_{(AcAlaXAlaNHtButyl)} - \log P_{(AcAlaGlyAlaNHtButyl)}$.

b Data of Fauchere and Pliska determined from the octanol-water partition coefficients of N-acetyl amino acid amides; $\pi = \log P_{(N\text{-acetyl amino acid amide})} - \log P_{(N\text{-acetyl glycyl amide})}$(Fauchere & Pliska, 1983).

c Retention coefficients in C18 reverse phase HPLC obtained in the present study using the tripeptides at pH 2.

d Retention coefficients determined by Parker and coworkers (1986) at pH 7.

e Retention coefficients determined by Guo and coworkers (1986) at pH 2.

f Kyte and Doolittle's hydropathy scale (Kyte & Doolittle, 1982).

g Engelman and coworkers hydrophobicity scale (Engelman et al., 1986).

h. The π values calculated by Abraham and Leo (1987) using the fragment method. The values in the parenthesis are those calculated using full polar proximity effect.

i. The π values calculated by Roseman (1988) using the fragment method.

j The π values calculated by Akamatsu and coworkers (1989) using the fragment method.

k The free energy of transfer of side chains from water into octanol was calculated according to the equation, $\Delta Gx = -2.3\ RT\ \pi$

Table III (continued)

using the π values of this study.

[l] The free energy of transfer of side chains from water into octanol was calculated according to the equation, ΔGx = -2.3 RT π using the π values of Fauchere and Pliska (1983). and's (c').

[m] Parker and coworkers retention coefficients data were converted into the free energy term by assuming ΔGx of Ala to be -0.42 to directly compare with data of Fauchere and Pliska.

[n] K&D data was scaled to set value for Gly to be 0. The sign was changed to be consistent with other data.

[o] ESG data was scales to set value for Gly to be 0.

[p] The free energy of transfer of side chains from water into octanol was calculated according to the equation, ΔGx = -2.3 RT π using the π values of Abraham and Leo (1987). For His, Asp and Glu π values obtained using the full polar proximity effect were used.

[q] The free energy of transfer of side chains from water into octanol was calculated according to the equation, ΔGx = -2.3 RT π using the π values of Roseman (1988).

[r] The free energy of transfer of side chains from water into octanol was calculated according to the equation, ΔGx = -2.3 RT π using the π values of Akamatsu and coworkers (1989).

[s] Correlation coefficient between the indicated data and the π values obtained in this study from the octanol water partition coefficients of tripeptides to other data.

[t] Correlation coefficients between the indicated data and the present result with data for Asp and Glu omitted.

Correlation coefficients between the result of the present study and others with or without Asp and Glu residues are included in the last two columns. The π values obtained from the present study as described in the preceding section are presented in the first row.

The relative order of hydrophobicity of non polar residues agreed well among the studies (Table III). However a detailed comparison between our data and the various studies reveals some significant differences. The correlation of our data to the F & P scale was 0.86 and improved when Asp and Glu were not included (The last column in the Table III). The difference between the hydrophobicity values of Asp and Glu obtained in the present study and those of Fauchere and Pliska (1983) is more than 2 Kcal/ mol in terms of free energy of transfer. Abraham and Leo (1987) suggested that the interaction between terminal amide group and the carboxyl group in N-acetyl aspartyl amide or N-acetyl glutamyl amide might contribute to the increased hydrophobicity measured for Asp and Glu by Fauchere and Pliska. A similar interaction such as a hydrogen bond between the peptide backbone and the carboxylate group of Asp or Glu is sterically possible in the tripeptides containing Asp and Glu. Whether such an interaction is less favorable in tripeptides than in N-acetyl aspartyl amide or N-acetyl glutamyl amide is not known.

The hydrophobicity values of Ala, Phe, Trp and Pro residues determined from the partitioning of tripeptides were less than those obtained from N-acetyl amino acid amides (Fauchere & Pliska, 1983). The π_X value is a true measure of the hydrophobicity of the side chain X only when the model compounds are the same in all aspects except the substitution of hydrogen in the parent compound with the group of interest X. If the substituent group influences the conformation of the model compounds, the π value may reflect the influence of conformation on steric effects, hydrogen bonding, or electronic effects (Leo et al., 1971). In the case of non polar N-acetyl amino acid amides, conformation is unlikely to differ significantly among the derivatives. The tripeptide AcAlaGlyAlaNHtButyl, which served as the reference compound in this study might form a folded structure more easily than other tripeptides due to the inherent flexibility of Gly

residue. The first and the last peptide bonds in the folded tripeptide could then participate in a hydrogen bond which would increase the log P of the peptide AcAlaGlyAlaNHtButyl. Hence the π for other residues would be reduced resulting in the discrepancy observed for the π values for non polar residues between the present study and Fauchere and Pliska (1983). Although we did not examine the peptide conformation in this work, NMR studies of tetrapeptide (Bundi & Wuthrich, 1979) Gly-Gly-X-Ala in water indicated that the tetrapeptides adopted a random coil structure regardless of the nature of residue X. So we think it is unlikely that the folded conformation of AcAlaGlyAlaNHtButyl is a major factor in the discrepancy in the π values between our values and those Fauchere and Pliska.

NMR indicates the bulky side chains are located near the peptide backbone rather than directed away from the peptide backbone into the solvent. The non random spatial arrangements of the bulky hydrophobic residues was attributed to intramolecular short range interactions independent of chain length (Bundi & Wuthrich, 1979). The non polar side chains of tripeptides used in the present study would prefer to decrease the contact with water similar to the tetrapeptides discussed above. An intramolecular hydrophobic interaction between adjacent non polar residues would stabilize the peptide in water and this would be reflected as lower partitioning into octanol. Therefore the apparent hydrophobicity of individual residues in tripeptides calculated from the difference of log P between AcAlaXAlaNHtButyl and AcAlaGlyAlaNHtButyl would be lower than π values obtained from N-acetyl amino acid derivatives. Lower π values due to the folding or intramolecular hydrophobic interaction have been reported for other organic compounds (Leo et al., 1971).

The experimental data of log P of tripeptides reported by Akamatsu and coworkers (1989) also suggests that the hydrophobic contribution of bulky side chains in tripeptides appear to be less than that in N-acetyl amino acid amide derivatives. The side chain contribution of residues in tripeptides is compared to the corresponding N-acetyl amino acid amide data (Table IV). The difference of log P between Phe-Val-Gly and Phe-Val-Ala

Table IV. Comparison of hydrophobicity contribution of side chains in tripeptides and N-acetyl amino acid amides.

Ala	[a] $\log P_{(Phe\text{-}Val\text{-}Ala)} - \log P_{(Phe\text{-}Val\text{-}Gly)} = 0.14$	
	[b] $\log P_{(AcAlaAlaAlaNHtButyl)} - \log P_{(AcAlaGlyAlaNHtButyl)} = 0.09$	
	[c] $\log P_{(N\text{-}Ac\text{-}Ala\text{-}amide)} - \log P_{(N\text{-}Ac\text{-}Gly\text{-}amide)} = 0.31$	
Phe	[a] $\log P_{(Phe\text{-}Phe\text{-}Phe)} - \log P_{(Gly\text{-}Phe\text{-}Phe)} = 1.31$	
	[b] $\log P_{(AcAlaPheAlaNHtButyl)} - \log P_{(AcAlaGlyAlaNHtButyl)} = 1.61$	
	[c] $\log P_{(N\text{-}Ac\text{-}Phe\text{-}amide)} - \log P_{(N\text{-}Ac\text{-}Gly\text{-}amide)} = 1.79$	
$CHCH_3$	[a] $\log P_{(Leu\text{-}Val\text{-}Leu)} - \log P_{(Leu\text{-}Ala\text{-}Leu)} = 0.46$	
	[c] $\log P_{(N\text{-}Ac\text{-}Val\text{-}amide)} - \log P_{(N\text{-}Ac\text{-}Ala\text{-}amide)} = 0.91$	

[a] Partition coefficients of tripeptides measured by Akamatsu and coworkers (1989).

[b] Partition coefficients of tripeptides measured in the present study.

[c] Partition coefficients of N-acetyl amino acid derivatives measured by Fauchere and Pliska (1983).

was only 0.14 compared to 0.31 in the N-acetyl amino acid amide. The difference of log P between Phe-Phe-Phe and Gly-Phe-Phe was 1.31 compared to 1.79 in N-acetyl amino acid amide. The difference between Val and Ala in tripeptides was only a half of that in N-acetyl amino acid amides. The di- and tripeptides used by Akamatsu and coworkers were composed of rather bulky side chains such as Val, Leu, Phe or Trp. It is likely that intramolecular interactions between adjacent residues would decrease the total hydrophobic surface area available to water molecules and decrease the apparent hydrophobicity of individual residues.

Our results and those of Akamatsu and coworkers (1989) support the idea that the apparent hydrophobicity of side chains in peptides is lower than that determined using N-acetyl amino acid amides due to the interaction between adjacent residues in the peptide sequence. Therefore the π values obtained using peptides should represent a better estimate of side chains hydrophobicity in peptides. The magnitude of the intramolecular hydrophobic interaction in peptides would depend upon the nature of the residues involved. For the blocked tripeptides, both the tButyl group and the Ala residues could be involved in such hydrophobic interaction. The interaction with the tButyl group might be similar to that of a valine due to their similar size. If the π values were measured using peptides of the sequence Gly-X-Gly instead of AcAlaXAlaNHtButyl, they might be a little greater than the values obtained in this study. Whereas if Leu-X-Leu were used, π values might be smaller than the present results.

Besides the direct measurement of partition coefficients, retention coefficients in reverse phase HPLC has been an alternative way to determine the hydrophobicity of solutes. The retention coefficients of tripeptides measured in this study are presented in Table III. Chromatograms were obtained using a gradient of methanol and water containing 0.1 % of trifluoroacetic acid where the pH of aqueous phase was about 2. The correlation between $P_{o/w}$ of acetylated tripeptides and the capacity factor on C18 column is illustrated in Figure 2. The Po/w data of AcAlaAspAlaNHtButyl and AcAlaGluAlaNHtButyl are those

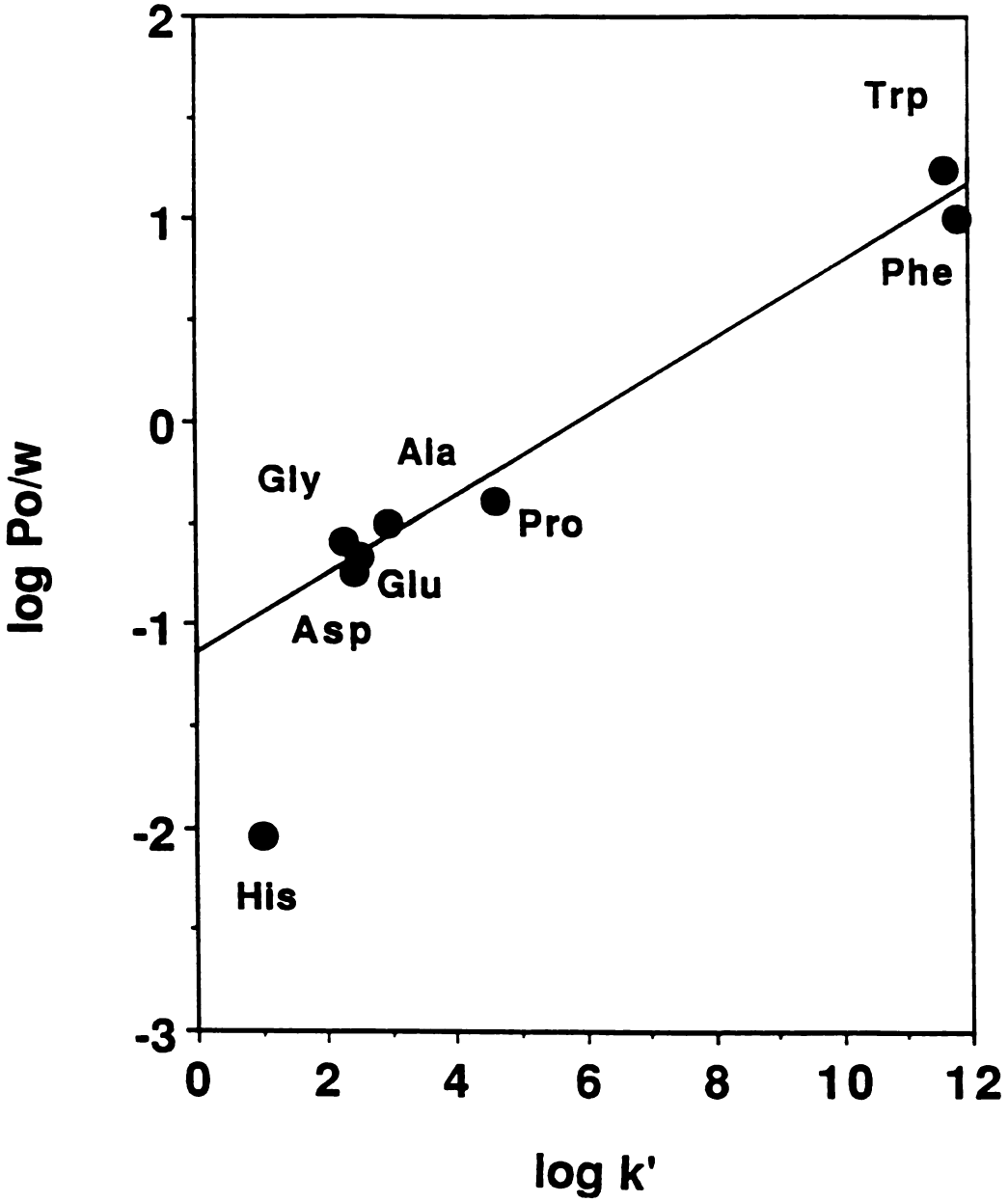


Figure 2. Correlation between the partition coefficients ($P_{o/w}$) and the capacity factor (k') of tripeptides. The capacity factor k' was calculated from the retention time on reverse phase HPLC. $k' = (t - t_o)/t_o$ where t is the retention time of the solute and t_o is that of solvent. HPLC eluent was methanol and water containing 0.1 % TFA and pH was less than 2. Therefore The $P_{o/w}$ data at pH 2 were used for the Asp and Glu containing peptides and data at pH 3 for the His peptide.

measured at pH 2 and data of AcAlaHisAlaNHtButyl is that measured at pH 3 because HPLC was run at pH 2. If the data for AcAlaHisAlaNHtButyl is excluded the linear correlation is excellent (R = 0.98). The positively charged AcAlaHisAlaNHtButyl might form an ion pair with trifluoroacetate and hence have a longer retention time than the uncharged peptides which under the same conditions cannot form ion pairs (Guo et al., 1987). The good correlation between the retention time on the C18 column and the $P_{o/w}$, suggests that both methods are measuring a relative hydrophobicity value. Therefore the $P_{o/w}$ values measured here are a further validation of reverse phase HPLC as a method to measure relative hydrophobicity of peptides. The retention coefficients in reverse phase HPLC might be expected to be more sensitive to the size or bulkiness of the solutes than the octanol-water partition coefficients due to the more ordered array of C18 chains on the reverse phase column. However our results do not reveal any significant difference between the two methods. It might be that solutes of larger molecular weight are required before the effect of chain order is observed.

The hydrophobicity values derived by Parker and coworkers (PGH scale) (Parker et al., 1986; Guo et al., 1986) were based upon reverse phase HPLC retention time of peptides, Ac-Gly-X-X-$(Leu)_3$-$(Lys)_2$-amide, where X was substituted by the 20 amino acids. The contribution of the side chain X was calculated by subtracting the retention time of the core peptide Ac-Gly-$(Leu)_3$-$(Lys)_2$-amide and dividing the result by two. The correlation between the retention coefficients determined in the present study and the PGH scale obtained at pH 2 (Guo et al., 1986) was 0.964. The correlation of our π values obtained from partitioning measurement of tripeptides with PGH scale at pH 7 was 0.83 and improved to 0.91 when the Asp and Glu data were not included. Aspartic acid and Glutamic acid are more hydrophilic in our study than in the PGH scale (Parker et al., 1986). Interestingly the correlation (R = 0.98) between the π values obtained at pH 2 in the present study and PGH scale at pH 2 (Guo et al., 1986) was better than at pH 7 and there was no effect of eliminating (R = 0.98) Asp and Glu residues from the comparison.

The unusually high hydrophobicity of Asp and Glu in PGH scale at pH 7 compared to others may be due to the electrostatic interaction between the negatively charged Asp and Glu with positively charged lysine in the sequence Parker and coworkers used. If one Glu and Lys interacted and neutralized the charge then the difference of retention time between Ac-Gly-Glu-Glu-$(Leu)_3$-$(Lys)_2$-NH_2 and Ac-Gly-$(Leu)_3$-$(Lys)_2$-NH_2 divided by two cannot be an appropriate hydrophobicity for Glu because the core peptide contains two positively charged Lys whereas only one Lys would be positively charged in the peptide containing Glu. The same would be true for the Asp containing peptides and the retention coefficients of Asp and Glu obtained using this sequence would underestimate the hydrophilicity of Asp and Glu residues.

Parker and coworkers compared their data with others by arbitrarily setting the hydrophobicity of the most hydrophobic residue of each scale +10 and the most hydrophilic residue -10. This comparison does not give information on absolute values of hydrophobicity of residues because the most hydrophobic and the most hydrophilic residues differed depending on the hydrophobicity data. Furthermore the most hydrophobic and hydrophilic residues are most likely to have some form of intramolecular interaction. A more appropriate comparison might be to set the hydrophobicity of Gly and Ala residue first, because the hydrophobicity of these residues are more consistent among studies. When the retention coefficients in the PGH scale at pH 7 were converted to a Kcal/mol unit by setting the Ala residue to -0.42, the value from Fauchere and Pliska (1983) (Table III) , the values for Phe and Trp are significant lower than those obtained by Fauchere and Pliska. This may be due to the intramolecular hydrophobic interaction discussed above or the effect of ordered structure in the C18 column or combination of both.

The Kyte and Doolittle scale (K & D scale) (Kyte & Doolittle, 1982) was derived using a combination of water-vapor partitioning data of amino acid side chain analogs (Wolfenden et al., 1981) and the partitioning of side chains between the protein surface and

the interior (Chothia, 1976). This scale has been widely used in locating putative membrane spanning regions in membrane proteins. The correlation between our data and the K & D scale was 0.46 and did not improve when polar residues were excluded. The poor correlation between the K & D scale and other hydrophobicity scales has been discussed previously (Engelman et al., 1986). A large factor in the poor correlation between our data and the K & D scale is the use of the vapor phase as a reference phase in Wolfenden (1981). Hydrogen bonds cannot form in the vapor phase with polar residues, whereas they can in octanol phase (Engelman et al., 1986).

Engelman and coworkers (1986) developed a hydrophobicity scale (ESG scale) by summing the hydrophobic and hydrophilic components for the free energy of water to oil transfer of each amino acid. The hydrophobic component was calculated from the surface area of side chains and the fact that the hydrophobic free energy is linearly correlated with the water accessible surface area(20-25 cal/mol/$Å^2$). The hydrophilic component was based upon the transfer energy of the polar groups from water to oil as determined from various model compounds. The correlation of our data with ESG scale was 0.87. However when the data for Asp and Glu were excluded, the correlation was 0.39. In this case, the poor correlation for the hydrophobic residues is due to the addition of an excessive hydrophilic component to the Trp and His residue in the ESG scale.

Two major findings from the above comparison can be summarized as follows: (1) The hydrophobicity of non polar side chains in a peptide structure is less than in amino acid analogues. This is attributed to intramolecular hydrophobic interactions. (2) Other discrepancies which cannot be explained by intramolecular interactions are as follows: The differences between our data and the K&D or ESG scale, 1 Kcal/mol for Pro, 2 Kcal/mol for Trp and 3 Kcal/mol for His, seem to be due to the inappropriate assignments of those values in K&D or ESG scale as discussed above. For Asp and Glu, the difference between our data and that of Fauchere and Pliska was 2 Kcal/mol and it was about 6 Kcal/mol when

compared to the ESG value. It is not clear at the present why such a large discrepancy among various scales exists for the hydrophobicity values of Asp and Glu.

A 2 Kcal/mol difference in side chain hydrophobicity results in a 600 fold difference in octanol-water partition coefficients (P) between the Gly and Trp containing tripeptides. Its effect is also clearly evident in the measured partition coefficient of the tripeptides into dimyristoylphosphatidylcholine liposomes. The measured partition coefficients of AcAlaPheAlaNHtButyl and AcAlaTrpAlaNHtButyl into dimyristoylphosphatidylcholine liposomes were 86 and 482, respectively while partitioning of the other tripeptides was not detectable. The impact of using inappropriate hydrophobicity values for residues will of course depend upon the length and composition of the peptides. However in a short peptide or when a large fraction of the residues are assigned inappropriate hydrophobicities the final prediction could be considerably misleading.

COMPUTATIONAL METHODS TO ESTABLISH AMINO ACID HYDROPHOBICITIES

An alternative approach to establishing amino acid hydrophobicities is to divide or fragment the side chain into chemical groups and to sum the hydrophobic contribution from each group to obtain a π value for the side chain. The amino acid hydrophobicity calculated using the fragment method by three independent workers are listed in the Table III. The π values for Ala and other aliphatic side chains (not shown in the Table) do not show significant difference. However, π values of the aromatic side chains differ depending upon the analogs or fragments chosen by each author. Histidine, aspartic acid and glutamic acid also showed large differences between the A&L (Abraham & Leo, 1987) and Roseman (1988) scales. Roseman calculated the π values for protonated Asp (–0.71) and Glu (– 0.18) from the log P of protonated acetic acid and propionic acid, respectively. The π values obtained in our study for Asp and Glu at pH 2 were – 0.14 and – 0.08

respectively. Roseman calculated the π values of Asp and Glu at pH 7 assuming that the difference between the ionized and the protonated form is 4.06 which is the average value for an aliphatic acid. However, our data on the pH dependence of log P of tripeptides containing Asp or Glu showed that the difference of log P between ionized and protonated forms was about 2.6. As a result the difference between our data and the Roseman π values at pH 7 was larger than for the protonated forms. Abraham and Leo (1987) calculated the hydrophobicity of these residues by summing the contribution of each fragment and correcting for the polar proximity effect. In their π values, aspartic acid is more hydrophobic than glutamic acid. As discussed above, this is due to the difficulty in assigning an appropriate value for the polar proximity effect.

Akamatsu and coworkers (1989) attempted to predict the log P of di- and tripeptides from the sum of π values of side chains. Using the π values calculated by Akamatsu and coworkers (Table III), they obtained the following relationship for tripeptides between the experimentally observed log P and the sum of π: $\log P' = 0.804 \Sigma \pi - 5.114$ ($R = 0.892$). To improve the fitting, they introduced the structural parameter $E_s^{c'}$, which was originally developed to explain the steric effect in the rate of ester hydrolysis. The $E_s^{c'}$ parameters improved the fitting between the predicted and measured partition coefficients. The authors proposed that the solvation of peptide bond -CONH- and the terminal amino and carboxylate groups is influenced by the side chains and the polarity of those polar groups would be further reduced by bulky side chains. Since π values are computed from the difference of log P between the reference (Gly derivatives) and the experimental compounds, the steric effect of bulky residues on the peptide bond will be normalized whether they are in the tripeptides or N-acetyl amino acid amides. For example the effect of Trp on the peptide bond in N-acetyl tryptophanyl amide compared to N-acetyl glycyl amide should be the same as that in Ala-Trp-Ala compared to Ala-Gly-Ala. Therefore the reduced polarity of the peptide backbone by bulky side chains cannot be the explanation for

the apparently smaller contribution to the hydrophobicity of non polar residues in tripeptides .

Akamatsu and collegues used a number of different equations to predict the log P. The equation 7 in their paper include the sum of π values of Fauchere and Pliska, steric parameters and the correction factors for each residues: $\log P' = 1.067 \Sigma \pi + 0.647 E_s^{c'}(R_N) + 0.454 E_s^{c'}(R_M) + 0.322 E_s^{c'}(R_C) + 0.345 I_Y - 0.159 I_W + 0.231 (I_s + I_T) - 4.744$. The exact coefficients for the steric parameter and the correction factor for each residue depended upon the π values used. The equation for the prediction of log P using π values of Akamatsu and coworkers is shown in the Table V. The log P values of tripeptides experimentally determined and those calculated by Akamatsu's equation with or without steric effect ($\log P = \Sigma \pi_i - 4.7$) are shown in Table V. The $\Sigma \pi_i$ represents the sum of contributions of the side chains; therefore -4.7 can be regarded as the log P of the tripeptide backbone alone. The last three columns show that calculated log P values differed significantly depending upon the π values used. The log P values calculated using the π values obtained in this study (the last column in Table V) were as good as the log P obtained by Akamatsu using the steric parameter and the correction factor in addition to $\Sigma \pi$.

In summary the fragment method is useful to predict the log P of compounds where complicated intramolecular interaction are absent. In peptides or aromatic or ionizable compounds intramolecular interactions and the polarity of the compound may present obstacles to the accurate prediction of ΔG of transfer.

Table. V. Partition coefficients of tripeptides

Peptides	log Pexpt[a]	log Pcalc[b]	log Pcalc[c]	log Pcalc[d]	log Pcalc[e]
Gly-Phe-Phe	-1.33	-1.34	-0.84	-1.16	-1.52
Phe-Phe-Phe	-0.02	0.03	1.11	0.63	-0.09
Trp-Gly-Gly	-2.72	-2.83	-2.78	-2.45	-2.84
Trp-Phe-Ala	-1.00	-1.01	-0.55	-0.39	-1.18
Phe-Val-Ala	-2.19	-2.14	-1.20	-1.42	-1.82
Tyr-Gly-Phe	-1.86	-2.09	-1.59	-1.99	-

[a] Partition coefficients experimentally measured by Akamatsu and coworkers (1989).

[b] log P estimated from the equation 6 of Akamatsu and coworkers; $\log P' = 0.960\,\Sigma\pi + 0.561\,E_s^{c'}(R_N) + 0.338\,E_s^{c'}(R_M) + 0.255\,E_s^{c'}(R_C) + 0.164\,I_Y + 0.351\,I_W + 0.637\,I_M + 1.666\,(I_s + I_T) - 4.788$.

[c] log P calculated from the equation $\log P = \pi - 4.7$ using the π values of Akamatsu and coworkers (1989).

[d] log P calculated from the equation $\log P = \pi - 4.7$ using the π values of Fauchere and Pliska (7).

[e] log P calculated from the equation $\log P = \pi - 4.7$ using the π values obtained in this study.

CONCLUSIONS

Although there was a good correlation in the relative order, the hydrophobicity values of amino acid side chains in tripeptides were different from those measured in N-acetylamino acid amides (Fauchere & Pliska, 1983) or calculated using the fragment method (Abraham & Leo, 1987; Roseman, 1988; Akamatsu et al., 1989). Nonpolar amino acids such as Ala, Phe, Trp, Pro had a lower apparent hydrophobicity in the tripeptide than as N-acetylamino acid amides. The ionizable residues Asp and Glu in the tripeptide were about 2 Kcal more hydrophilic than as the N-acetyl amino acid amide (Fauchere & Pliska, 1983). The π values obtained in the present study using blocked tripeptides should represent a better estimate of the hydrophobicty of amino acid side chains in peptides than previous studies because they incorporate interactions between adjacent residues.

Based upon these studies, we propose that the free energy of transfer from water to octanol for the 8 amino acid side chains at pH 7.2 are 0, -0.13, -2.19, -2.52, -0.29, -0.16, 3.50 and 3.12 Kcal/mol for Gly, Ala, Phe, Trp, Pro, His, Asp and Glu, respectively.

CHAPTER 4. THERMODYNAMICS OF TRIPEPTIDE PARTITIONING

INTRODUCTION

The temperature dependence of the free energy of transfer of amino acid side chains is of central importance in the study of protein folding and protein thermal stability (Tanford, 1970; Edelhoch and Osborne, 1976; Schellman, 1987; Privalov, 1989; Makhatadze and Privalov, 1990). The measurement of protein thermal transitions can provide the computation of ΔH, ΔS and ΔG as a function of temperature, as well as heat capacity change (ΔC_p). These numbers can be used to establish the relative stabilities of proteins (Schellman, 1987). ΔC_p is considered a measure of the contribution of hydrophobic interactions to the stability of a protein. However the heat capacity increase accompanying protein denaturation is not well understood. This is partially due to the absence of information on the thermodynamic properties of amino acid residues (Makhatadze and Privalov, 1990).

The heat capacity of the amino acid side chains in aqueous solution have been measured by calorimetric methods (Tanford, 1970; Makhatadze and Privalov, 1990). The temperature dependence of transfer of hydrocarbons from pure hydrocarbons to water has also been used as a model for the temperature dependence of the hydrophobic interaction in protein stability (Baldwin, 1986). However this might not be the most appropriate reference phase for proteins. Information is absent for the heat capacity change following transfer from water into an octanol phase although octanol water partition coefficients of amino acids (Yunger and Cramer, 1981) or their analogues (Fauchere and Pliska, 1983) are frequently used as a measure of amino acid hydrophobicity. In this chapter, the temperature dependence of octanol-water partition coefficients of tripeptides are described. A linear and non linear van't Hoff analysis are used to obtain the thermodynamic parameters, ΔH, ΔS and ΔC_p for the transfer process of tripeptides from water into octanol.

The data are compared to the hydration heat capacity determined by previous workers. The heat capacity change for the transfer of tripeptides from the gas phase into the octanol phase is then estimated.

MATERIALS AND METHODS

PARTITION COEFFICIENTS

Samples for partition coefficients measurements were prepared as described in the previous chapter. Equilibration was obtained by one of the following two methods. The first method consisted of rotation on a rotator (Sepco Tube Rotator, Scientific Equipment Products, Baltimore, MD) and centrifugation to separate the phases after equilibrium had reached. In the case of the 4 °C measurement, samples were equilibrated while rotating for two days in a cold room and at higher temperatures (35, 45, 55 °C), the rotator was placed in an incubator which maintained the desired temperature ± 1°C. After one day of rotation, tubes were centrifuged for 15 minute and 1 or 1.5 ml of both phases were transfered into 20 ml scintillation vials and the radioactivity was determined. The second method was to vortex for 30 seconds after an one hour incubation in a water bath at the desired temperature. The sample tube was incubated another hour and vortexed again. After vortexing, samples were left overnight in a water bath to allow the phases to separate. The centrifugation step was omitted. Radioactivity was determined in both the octanol and the buffer phase. The partition coefficient ($P_{o/w}$) was calculated from the ratio of the DPM in the octanol phase to DPM in the buffer phase.

At room temperature the difference between partition coefficients measured by the two methods were less than 5 % for all the peptides studied. Partition coefficients of AcAlaTrpAlaNHtButyl measured by the two methods were the same up to 65 °C. At 45 and 55 °C, the difference was almost two fold for the ionized peptides such as

AcAlaAspAlaNHtButyl (pH 9), AcAlaGluAlaNHtButyl (pH 9) and AcAlaHisAlaNHtButyl (pH 3). The differences found for the ionized peptides might be due to the very low partition coefficients (1 x 10^{-4} to 10^{-3}) which would be much more sensitive to the amount of water present in octanol phase. Curve fitting of the data to estimate the thermodynamic parameters for these ionized peptides was not attempted. Details of the data analysis for the other peptides are described in the Results and Discussion.

Recovery of peptides from both phase was always in the range of 97 to 107 % indicating no significant peptide adsorption onto the glass. Similar peptide recoveries were obtained using silanized glassware.

RESULTS AND DISCUSSION

TEMPERATURE DEPENDENCE OF PARTITIONING OF TRIPEPTIDES

The free energy of transfer (ΔG) of the various tripeptides from aqueous to octanol phase was calculated using the equation, $\Delta G = -2.3\ RT \log P$, where P is the partition coefficient calculated using the ratio of mole fraction of peptide in the octanol and aqueous phases. $P_{o/w}$, the ratio of molar concentration in octanol and buffer phases can be converted to P by multiplying the molar volume ratio of the two solvent so that $P = P_{o/w}\ V_{oct}/V_{water} = 8.748 \times P_{o/w}$ assuming the molar volume ratio of the two solvents remains the same over the temperature range studied.

The temperature dependence of P of six tripeptides at pH 7.2 is illustrated in Figure 1. The error bars are smaller than the symbols. Non-linearity above 45 °C is obvious in the data for Phe and Trp peptides. To rule out the possibility of chemical degradation of AcAlaPheAlaNHtButyl and AcAlaTrpAlaNHtButyl at high temperature, the partition coefficients of these two peptides were measured at 65 °C and an aliquot of the buffer phase was re-equilibrated with fresh octanol at room temperature. For both peptides, P at

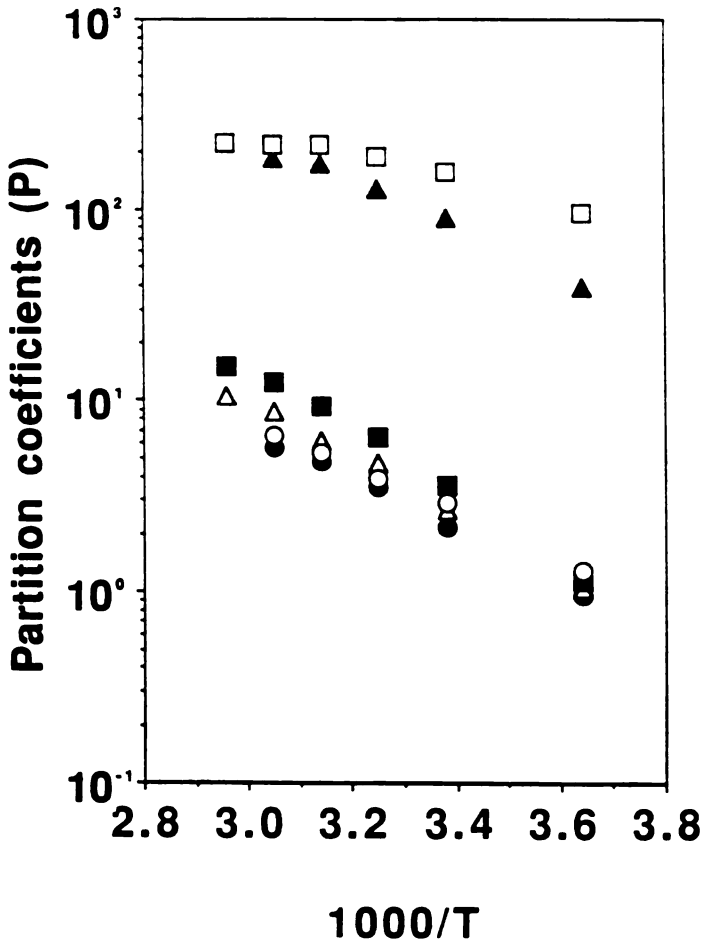


Figure 1. Temperature dependence of partition coefficients of tripeptides AcAlaXAlaNHtButyl where X was Trp (□), Phe (▲), Pro (■), His (△), Ala (○) and Gly (●). P is the the partition coefficient calculated from the ratio of the peptide mole fraction in the octanol versus the water phase at pH 7.2. Each symbol represents the mean of triplicate measurements and the standard deviation error bars are smaller than symbols.

room temperature following incubation at 65 °C was the same as P measured at room temperature without preexposure to 65 °C. This suggests that the peptides were intact after the one day equilibration at 65 °C.

The thermodynamic parameters were computed from the data using the Apple Macintosh program MINIM. In the linear analysis, the enthalpy (ΔH) and entropy (ΔS) of transfer are assumed to be temperature independent. ΔH is calculated using the linear van't Hoff relationship:

$$d \ln P/dT = \Delta H(T)/ RT^2.$$

When ΔH is independent of temperature,

$$d \ln P/d (1/T) = -\Delta H/R.$$

Therefore, ΔH can be estimated from the slope of log P vs. 1/T. Once ΔG and ΔH are known ΔS can be calculated from the relationship $\Delta G = \Delta H - T\Delta S$. The calculation assumes that the mutual solubility of the two solvents and the molar volume of the solvents remain the same in this temperature range.

When curvature is evident in the van't Hoff plot, it has been diagnostic for a hydrophobic interaction (Tanford, 1970). This indicates a heat capacity change (ΔC_p) and a temperature dependence of ΔH (Tanford, 1970; Baldwin, 1986; Ha et al., 1989; Murphy et al., 1990; Dill, 1990). When ΔC_p is not dependent on temperature, $\Delta H(T)$ and $\Delta S(T)$ are defined as following:

$$\Delta H(T) = \Delta H_o + \Delta C_p(T-T_o).$$

$$\Delta S(T) = \Delta S_o + \Delta C_p \ln(T/T_o).$$

$$\Delta G = \Delta H(T) - T\Delta S(T).$$

Then,

$$\Delta G = \Delta H_o + \Delta C_p (T - T_o) - T\Delta S_o - T \Delta C_p \ln(T/T_o).$$

The second method of data analysis fits the parameters, ΔH_o, ΔS_o and ΔC_p by a non linear fitting of ΔG and T in the above equation. The ΔH_o and ΔS_o are the enthalpy and entropy of transfer at a reference temperature T_o (296 °K).

The thermodynamic parameters obtained by the two methods of analysis are presented in Table I. The ΔH of transfer from water into octanol is positive for all the tripeptides listed in Table V indicating that their transfer into octanol is entropy driven. The correlation coefficients were always better for the non linear fit than the linear van't Hoff fit. Standard deviations of ΔH and ΔS were less for the non linear fit than the linear fit for Ala, Phe, Trp and Pro peptides. The relatively small standard deviation of ΔC_p also indicates that the non linear fit better describes the transfer of these peptides from water into octanol and their heat capacity decreases upon transfer. However for the peptides containing Gly or His, the non-linear fit to the data was not significantly better than the linear fit and the heat capacity change was not significant.

The temperature dependence of $P_{o/w}$ of the ionizable peptides was measured at pH values where the peptides would be predominantly uncharged. The plot of P vs. 1/T is shown in Figure 2. The thermodynamic parameters of transfer of ionizable peptides are given in Table I. For the AcAlaAspAlaNHtButyl (pH 2) and AcAlaGluAlaNHtButyl (pH 2) the linear van't Hoff fitting resulted in smaller standard deviations of ΔH and ΔS than the three parameter fit. ΔH is positive for all cases. When the ionized peptides are the dominant species, the partition coefficients were sensitive to the experimental procedure and values at elevated temperatures were difficult to obtain. Therefore curve fitting to obtain the thermodynamic parameters was not informative due to the absence of a data set over a sufficiently wide temperature range.

Table I. Thermodynamic parameters of transfer of tripeptides from water into octanol

AcAlaXAlaNHtButyl X=	[a]ΔG (Kcal/mol)	[b]fit	[c]ΔH (s.d.) (Kcal/mol)	[d]ΔCp (s.d.) (cal/mol°K)	[e]ΔS (s.d.) (cal/mol°K)	[f]R^2	[g]s.d. of res	[h]Δ^{w}gCp (cal/mol°K)	[i]Δ^{oct}gCp (cal/mol°K)
Gly	-0.46	lin	6.11(0.33)	-	22(1.1)	0.993	35	-	-
		non	6.32(0.31)	-51(34)	23(1.1)	0.997	24	146	95
Ala	-0.59	lin	6.76(0.25)	-	25(0.9)	0.996	33	-	-
		non	7.17(0.17)	-45(12)	26(0.6)	0.999	14	161	116
Phe	-2.65	lin	5.07(0.39)	-	26(1.3)	0.991	52	-	-
		non	5.74(0.23)	-73(17)	28(0.8)	0.999	19	195	122
Trp	-2.99	lin	2.44(0.41)	-	18(1.3)	0.979	55	-	-
		non	3.19(0.14)	-81(10)	21(0.5)	0.999	12	208	127
Pro	-0.75	lin	7.55(0.46)	-	28(0.5)	0.989	61	-	-
		non	8.36(0.19)	-88(14)	31(0.5)	0.999	17	158	70
His (pH7.2)	-0.63	lin	5.46(0.23)	-	20(0.7)	0.996	23	-	-
		non	5.61(0.20)	-36(22)	21(0.7)	0.998	15	141	105
Asp (pH2)	-0.27	lin	4.58(0.27)	-	16(0.9)	0.989	37	-	-
		non	4.82(0.38)	-26(28)	17(1.3)	0.990	32	-	-
Glu (pH2)	-0.36	lin	4.86(0.27)	-	18(1.3)	0.979	54	-	-
		non	5.31(0.51)	-49(38)	20(1.7)	0.986	44	-	-

Table I (continued)

His (pH9)	-0.66	lin	4.49(0.30)	-	17(1.0)	0.9888	40	-	-
		non	4.92(0.31)	-46(23)	19(1.4)	0.995	26	-	-

a. Free energy of transfer at room temperature calculated from the partition coefficient P (8.748 x Po/w) according to $\Delta G = -RT \ln P$.

b. Curve fitting methods to estimate the thermodynamic parameters described in Result and Discussion. 'lin' is the linear regression analysis using van't Hoff equation $d \ln P/d(1/T) = -\Delta H/R$. 'non' is using non linear regression analysis according to the equation $\Delta G = \Delta Ho + \Delta Cp\,(T - To) - T\Delta So - T\Delta Cp \ln(T/To)$.

c. Enthalpy of transfer at room temperature. Standard deviations are shown in the parenthesis.

d. Heat capacity change of transfer from water into octanol. Only 'non' method gives the estimates of ΔCp. Standard deviations are shown in the parenthesis.

e. Entropy of transfer at room temperature. Standard deviations are shown in the parenthesis.

f. Correlation coefficients for each regression analysis.

g. Standard deviation of residuals. The residuals are the difference between actual data points and the values estimated according to the regression curve.

Table I (continued)

h. Hydration heat capacity of tripeptides calculated according to the group additivity assumption using the values given in Makhatadze and Privalov (1990). Hydration heat capacity of tButyl group was assumed to be the same as that of Leu. When the value of Val is used instead of Leu, the calculated hydration heat capacity of tripeptides decreases by 10.

i. Heat capacity change of transfer of tripeptides from gas to octanol phase calculated using the equation $\Delta^{oct}{}_gC_p = \Delta^w{}_gC_p + \Delta C_p$. Hydration heat capacity of tButyl group was assumed to be the same as that of Leu. When the value of Val is used instead of Leu, the calculated hydration heat capacity and therefore $\Delta^{oct}{}_gC_p$ of tripeptides decrease by 10.

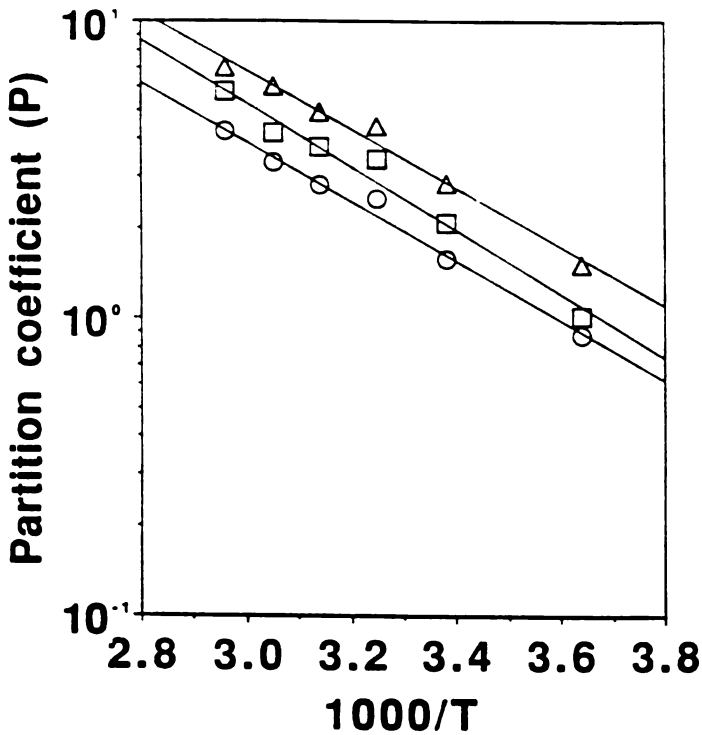


Figure 2. Temperature dependence of the partition coefficients of ionizable peptides, AcAlaHisAlaNHtButyl (△), AcAlaGluAlaNHtButyl (□) and AcAlaAspAlaNHtButyl (○) at a pH where the peptides are predominantly uncharged. Each symbol represents the mean of triplicate measurements and the standard deviation error bars are smaller than symbols.

RELATIONSHIP BETWEEN ΔH and ΔS

The ΔH of the Trp peptide is noticeably less than that of other peptides; the ΔH of the Phe peptide is also less than those of the Ala and the Pro peptides which had comparable ΔS values. One way to compare the data is to calculate ΔH/ΔS or to plot ΔH vs. ΔS. The ratio ΔH/ΔS of the peptides shown in Table I is around 270 except for the Phe and Trp peptides. The ratio was 200 for Phe peptide and 153 for Trp peptide. The difference is well illustrated in the plot of ΔH vs. ΔS (Figure 3). Phe and Trp peptides are obvious outliers.

Barclay and Butler (1938) discovered a linear relationship between the ΔH and ΔS of solution of various solutes when dissolved into into the same solvent. Frank and Evans (1945) explained the physical interpretation for this effect as follows: When a solute is vaporized, work must be done against the intermolecular forces between solute molecules. Since there are no intermolecular interactions in the vapor phase, the enthalpy of vaporization is positive. Upon going from the vapor phase into solution, solute-solvent interactions occur, and the stronger the intermolecular forces the more negative the entropy of solution will be. The solute-solvent interactions will also influence the reorientation of molecules in solution; the stronger the intermolecular forces the lower the entropy of the system. The stronger the intermolecular forces, the more negative is the enthalpy of solution. Barclay and Butler (1938) found that the slope dΔS/dΔH of solution of various solutes in water is twice that of solution into organic solvents. The difference in dΔS/dΔH between water and the organic solvents has been ascribed to the unique structured nature of aqueous solution, whereby water molecules organize around solutes.

The Barclay-Burtler relations for solution into water and solution into octanol can be written as follows:

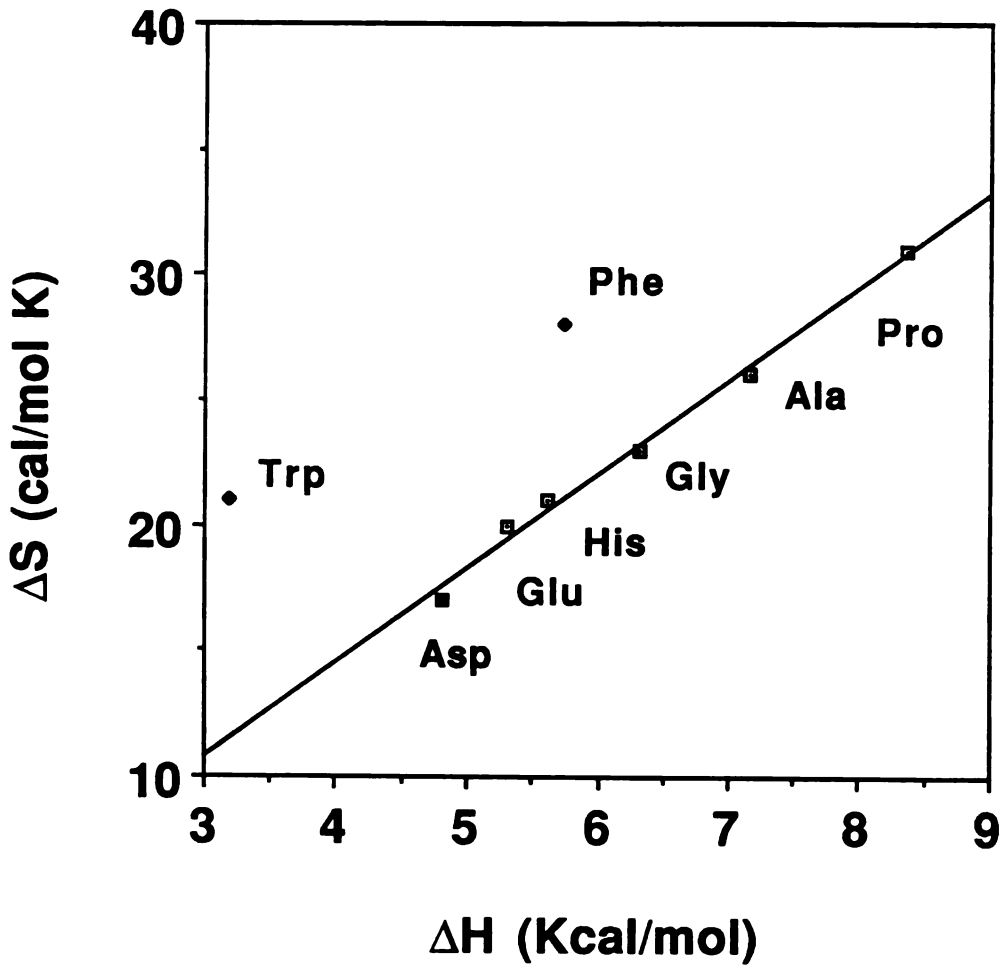


Figure 3. Entropy change versus enthalpy change for transfer of tripeptides from water into octanol. The data points of the Trp and Phe peptides were not included for the curve fitting.

$\Delta S_w = a_w + b_w \Delta H_w$ and $\Delta S_{oct} = a_{oct} + b_{oct} \Delta H_{oct}$.

Therefore the relationship between ΔH and ΔS of octanol-water partitioning can be written as follows (Katz and Diamond, 1974):

$$\Delta S = a_{oct} - a_w + \Delta H_{oct} (b_{oct} - b_w) + b_w \Delta H.$$

A linear relationship between ΔS and ΔH is expected when $\Delta H_{oct} (b_{oct} - b_w) << b_w \Delta H$ or ΔH_{oct} is linearly related to ΔH. The first will be true if $b_{oct} - b_w = 0$ or $\Delta H_{oct} << \Delta H$. For non polar solvents the thermodynamics of transfer are determined mainly by the interaction of the solutes with water (Davis et al., 1974). If that is also the case in octanol-water partitioning system, then $\Delta H_{oct} << \Delta H$ will be the reason for the observed linearity between ΔH and ΔS of octanol-water partitioning. The ΔH_w determination either by calorimetry, or by measurement of the solubility of peptides in water, as a function of temperature will answer the question since $\Delta H = \Delta H_{oct} - \Delta H_w$. If ΔH_w is similar to ΔH, the interaction of the solutes with water is the major factor determining the thermodynamics of octanol-water partitioning. The Barclay-Butler constant for solution into octanol b_{oct} is not known. The linear relationship between ΔH and ΔS in partitioning of small organic compounds between bilayers and water was attributed to the fact that the Barclay-Butler constants for water and dimyristoylphosphatidylcholine membranes were the same (Katz and Diamond, 1974).

One explanation for the outlying Phe and Trp peptides might be that interaction of aromatic rings with solvents is different from that of non aromatic compounds. Leo and coworkers (Leo et al., 1976) reported that the linear relationship between log $P_{o/w}$ and solute volume depended on the type of solutes. Aromatic hydrocarbons had lower $P_{o/w}$ compared to alkyl compounds of similar volume. They suggested that the difference was due to the orientation of water molecules around the cavity formed by the solute. Aromatic compounds are rich in π electrons and the π electrons can interact with the dipole of water. An alternative possibility is the poorer accommodation of aromatic compounds by octanol. The alkyl chains of octanol might accommodate alkyl solutes better than aromatic solutes.

They rejected the second possibility since benzene partitions into hexane as well as it does into itself. This argument is not compelling since benzene or hexane are isotropic solvents whereas octanol can have local structure due to the hydrogen bond among the hydroxyl groups (Figure 4 in Chapter 1).

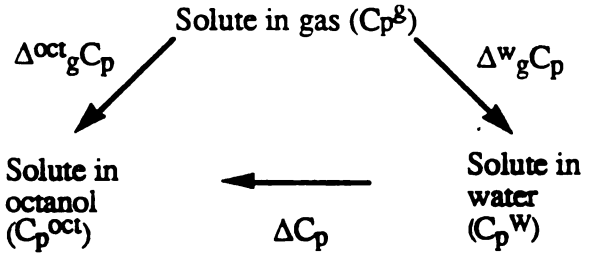
Returning to the question of the molecular mechanism for the smaller $\Delta H/\Delta S$ of Phe and Trp, the major factor is likely the interaction of π electrons with water. For the Trp peptide, an additional contribution might come from the interaction between the -NH group in the indole ring of Trp and the hydroxyl group of octanol. The partition coefficient of Trp was higher than that of Phe and other hydrophobic residues when octanol was used as the reference phase. In chloroform, cyclohexane, vapor phase and octanol supports the side chain analogue of Trp had a lower partition coefficient than Phe (Radzicka and Wolfenden, unpublished data).

The data point of the Pro peptide in Figure 3 seems to fit into either group of peptides. Cyclic hydrocarbons show higher water solubility than the corresponding alkyl chains of the same carbon number (Davis et al., 1974). This is due to the smaller surface area in contact with water. In fact when the free energy of transfer is plotted against solute surface area, cyclic hydrocarbons and chain molecules fall on one linear curve (Davis et al, 1974). Therefore the Pro peptide can be grouped together with the non aromatic peptides.

HEAT CAPACITY CHANGE UPON PARTITIONING OF TRIPEPTIDES

The thermodynamic parameters obtained from the temperature dependence of partitioning may not be as precise as that obtained using a calorimetric method (Privalov & Gill, 1988). However, in the absence of direct measurement of ΔC_p for the transfer of peptides from an aqueous to an organic solvent, the data are a useful starting point for those interested in the transfer of amino acid side chains between phases such as occurs in protein folding.

Recently Makhatadze and Privalov (1990) determined the hydration heat capacity ($\Delta^{w}_{g}C_p$) of amino acid side chains from calorimetric data of simple organic analogues of side chains and peptides. Direct comparison between their data and ours is not meaningful because the hydration heat capacity ($\Delta^{w}_{g}C_p$) is defined as the difference between the heat capacity of amino acid side chains in aqueous solution (C_p^{W}) and that in the gaseous phase (C_p^{g}). In the present study ΔC_p is the difference between the heat capacity of tripeptides in octanol (C_p^{oct}) and in aqueous solution (C_p^{W});



Therefore $\Delta C_p = C_p^{oct} - C_p^{W} = \Delta^{oct}_{g}C_p - \Delta^{w}_{g}C_p$, where $\Delta^{oct}_{g}C_p$ is the difference between the heat capacity of solute in the vapor phase and in octanol. It will be interesting to compare the ΔC_p and $\Delta^{w}_{g}C_p$ of amino acid side chains, the latter given by Makhatadze and Privalov (1990). Unfortunately the quality of the present data for AcAlaGlyAlaNHtButyl is not as good as other non polar peptides so we are unable to calculate the ΔC_p of the amino acid side chains with any assurance.

An alternative approach is to compute the hydration heat capacity ($\Delta^{w}_{g}C_p$) of tripeptides used in the present study. The $\Delta^{w}_{g}C_p$ values of amino acid side chains and constituents determined by Makhatadze and Privalov can be used to estimate $\Delta^{w}_{g}C_p$ of tripeptides according to the group additivity concept. Makhatadze and Privalov calculated the heat capacity of side chains using the group additivity concept when direct experiment was not suitable. The tripeptides AcAlaXAlaNHtButyl of the present study can be fragmented into 4 CH_3, 4 CONHCH, tButyl and R where R is the side chain of the central residue X. Therefore the hydration heat capacity of the tripeptide is calculated as follows.

$$\Delta^{w}_{g}C_p\ (\text{AcAlaXAlaNHtButyl}) = 3\ \Delta^{w}_{g}C_p(CH_3) + 4\ \Delta^{w}_{g}C_p(\text{CONHCH}) + \Delta^{w}_{g}C_p(R) + \Delta^{w}_{g}C_p(\text{tButyl}).$$

The hydration heat capacity value for the tButyl group is assumed to be between those of Val and Leu. Once the $\Delta^{w}_{g}C_p$ of the tripeptides is computed, the heat capacity change accompanying transfer from gas to octanol ($\Delta^{oct}_{g}C_p$) can be calculated according to $\Delta^{oct}_{g}C_p = \Delta^{w}_{g}C_p + \Delta C_p$. The results are presented in the last two column of Table I.

Values for $\Delta^{oct}_{g}C_p$ for organic compounds have not been reported. For some aromatic hydrocarbon gases, the heat capacity of solution in benzene or CCl_4 is zero (Tanford, 1970). Therefore the large positive $\Delta^{oct}_{g}C_p$ values for tripeptides are quite surprising. It is difficult to suggest a compelling explanation with the limited data. However, the large positive $\Delta^{oct}_{g}C_p$ values for tripeptides may be due to the high concentration (2.3 M, Leo et al., 1971) of water in water-saturated octanol. If the change in the molar volume of solvents upon mixing is negligible, the volume occupied by water in one liter of octanol solution will be 41 ml. Therefore specific portions of tripeptides might be in contact with water in water-saturated octanol. This could be especially likely if the hydroxyl groups of octanol form hydrogen bonds, and cluster around the water molecules (Figure 4, Chapter 1). In this case octanol is not isotropic in its local structure. Solutes, such as tripeptides, might orient in with their hydrophobic groups in the alkyl chain region and peptide bonds (CONH) in the water clusters. In doing so portions of the hydrophobic groups may be in water clusters. Hence, unlike in hydrocarbon solutions where the heat capacity of solution is zero, the heat capacity of solution in octanol may have contributions from both hydrocarbon-like and water-like interactions. Thus $\Delta^{oct}_{g}C_p$ may be positive albeit less than the hydration heat capacity.

It should be pointed out that the solvent properties of microscopic clusters of water in octanol is probably different from that of bulk water. If 41 ml of water in octanol behaved like bulk water, the partition coefficient of ionized peptides would be at least 0.041. The octanol-water partition coefficient of AcAlaGluAlaNHtButyl at pH 10 (Chapter 3) was 6.9

x 10^{-4} which was two orders of magnitude lower than what it would be if the water molecules in octanol were like bulk water.

The heat capacity of solution of hydrocarbons in water is proportional to the hydrocarbon surface area or number of solvating water molecules (Gill, 1985). Makhatadze and Privalov (1990) showed two distinct linear relationships between the heat capacity of amino acid side chains in aqueous solution and their non polar accessible surface area (ASA). Linear amino acid side chains such as Ala, Val, Leu and Ile fell on one line, ring-containing amino acid side chains such as Pro, Phe and Trp fell on another line. The linear relationship between the hydration heat capacity ($\Delta^{w}_{g}C_p$) of these residues and their non polar accessible surface area (ASA) is also shown in the $\Delta^{w}_{g}C_p$ of the tripeptides (Makhatadze and Privalov, 1990) (Table I). The $\Delta^{w}_{g}C_p$ of the Pro peptide is smaller than those of the Phe or Trp peptides. As is evident inTable I, the difference between the heat capacity of tripeptides in octanol and in aqueous solution, ΔC_p, is not linearly related to the surface area of the residues. The ΔC_p of the Pro peptide is similar to those of the Phe or Trp peptides despite the smaller surface area of the Pro residue, whereas the octanol-water partition coefficient of the Pro peptide is smaller than either of the Phe or Trp peptides (Figure 1). Therefore unlike the hydration heat capacity (Baldwin, 1986; Makhatadze and Privalov, 1990), ΔC_p cannot be used as a direct measure of solute hydrophobicity.

CONCLUSIONS

Partitioning of tripeptides from water into the octanol phase was an entropy driven process in the temperature range studied. For peptides containing Ala, Phe, Trp and Pro transfer from water into octanol was accompanied by a decrease in the heat capacity. The calculated heat capacity change for the transfer of these peptides from the gas phase to octanol was positive and larger than the heat capacity change reported for the transfer process of other organic compounds into aprotic solvents such as benzene or CCl_4. Two

differences between octanol and these aprotic solvent are the unique solvent property of octanol due to the hydrogen bonding of the hydroxyl groups and the high concentration of water in saturated octanol. The difference of solution heat capacity between octanol and aprotic solvents may be due to these differences in the solvent property.

CHAPTER 5. PREDICTION OF MEMBRANE SPANNING SEGMENTS

INTRODUCTION

Biological membranes are composed of both proteins and lipids. The dynamic interaction between the two components control and regulate the behavior of cells. Although electrostatic interactions play an important role (Honig et al., 1986; Weinstein et al., 1982; Boggs, 1983) in the behavior of membranes, the hydrophobic interaction is the most important force involved in lipid-protein interactions. Contrary to soluble proteins, few membrane protein structures are known due to the difficulty of obtaining suitable crystals. Bacteriorhodopsin and the bacterial photoreaction center are two membrane proteins whose three dimensional structures have recently been resolved (for review, see Rees et al, 1989; Popot et al, 1989). Consequently a great deal of effort has been made to deduce a three dimensional picture of membrane proteins from their amino acids sequences.

Segrest and Feldman (1974) first showed the existence of hydrophobic segments in membrane proteins of known sequence and proposed the possibility of identification of membrane-penetrating segments by finding clusters of hydrophobic regions. To define hydrophobicity they used the free energy of transfer of amino acids from ethanol to water measured by Nozaki and Tanford (Nozaki and Tanford, 1971). To propose a model for the bacteriorhodopsin structure, Engelman and coworkers (1981) looked for clusters of hydrophobicity and compared them to the regions that are inaccessible to proteolytic cleavage.

Rose and Roy (1980) showed that the distribution of hydrophobicity over the amino acid sequence of proteins could distinguish interior segments from exterior segments in globular proteins. In this approach the average hydrophobicity of a finite number of residues (window) is evaluated using a hydrophobicity scale. The analysis is performed

along the polypeptide sequence starting from N-terminal by moving the window progressively toward the C-terminal (moving segment approach, Figure 1).

Argos and coworkers (1982) and Kyte and Doolittle (1982) adopted the approach of Rose and Roy to locate putative membrane spanning regions. Amino acid hydrophobicity scales derived by these two groups have been described in Chapter 1 and Chapter 3. Kyte and Doolittle's amino acid hydropathy scale and moving segment approach is the most frequently used method to identify membrane spanning segments. However there has been criticism concerning their assignment of hydropathy to individual amino acids. For example Trp was ranked more hydrophilic than Gly.

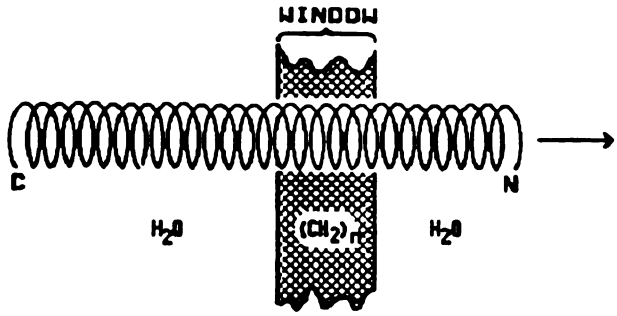
Several factors should be considered when using the moving segment approach and interpreting the result: (1) the hydrophobicity values assigned to each amino acids, (2) the length of a segment or a window, and (3) the position of midpoint line. These are the major factors influencing the outcome.

The importance of first factor can be examined by comparing the outcome using various hydrophobicity scales (Engelman et al., 1986) or arbitrarily setting values for certain residues (Hopp and Woods, 1981; Kyte and Doolittle, 1982).

The optimum length for the moving segment approach is controversial. Hopp and Woods (1981) reported that 6 residues was optimum to predict antigenic determinants whereas Kyte and Doolittle (1982) concluded that 7 to 11 residues produced the best result. For membrane proteins, Engelman and coworkers, considering the width of membrane bilayer, suggested that 20 residues was best.

For the midpoint line Kyte and Doolittle used the grand average of the hydrophobicity of amino acids found in sequenced proteins while Engelman and coworkers used a hydrophobicity value = 0 as the midpoint. This difference in the definition of the midpoint resulted in a discrepancy between their conclusions. Bacteriorhodopsin (BR) was predicted to have seven helices according to the analysis of Kyte and Doolittle (1982). However Engelman and coworkers concluded that the Kyte and Doolittle scale did not

(a)



(b)

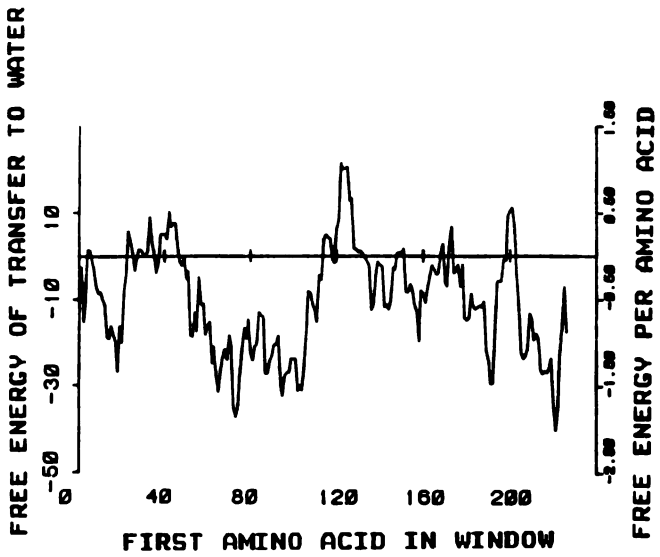


Figure 1. Prediction of membrane spanning segments (a) Schematic representation of the moving segment approach. The amino acid sequence represented as a helix is moved along the window of membrane bilayer interior. For each segment of the polypeptide chain in the window average free energy of transfer is calculated and the value is assigned to usually a central residue in that segment. The analysis is done progressively from N- terminal to C-terminal of the protein. (b) Hydropathy plot. The result of moving segment approach is represented as a plot of free energy of transfer of each residue versus the amino acid position. Taken from Engelman et al. (1984)

predict the membrane spanning helices of BR successfully. Engelman and coworkers conclusion seemed to be due to the inappropriate setting of the midpoint line because the shape of the two plots was similar.

Comparing the Kyte and Doolittle hydropathy plots of two subunits of the photoreaction center with the structure deduced from X-ray structural analysis, Michel and coworkers (Michel et al., 1986) found that the ends of the helices were predicted rather imprecisely although the five maxima of hydrophobicity corresponded roughly to the hydrophobic parts of the membrane-spanning helices. They suggested the presence of some polar or charged residues inside the membrane spanning segments related to their functional roles. Histidine in the membrane spanning region of the reaction center is known to be a ligand for iron (Fe^{+3}). Naturally the occurrence of charged residues in a charge neutralizing interaction will be a serious problem for any transmembrane sequence detecting program.

Several Pro are found in the membrane spanning region of bacteriorhodopsin. Amino acid hydrophobicity scales based upon the structural analysis of globular proteins assigned Pro a rather hydrophilic value due to the fact that Pro is often found at turns. To locate the membrane spanning regions, a more hydrophobic value for Pro, such as we have measured, would appear to be a more suitable choice.

To what extent a more representative hydrophobicity scale for the amino acids Pro, Glu, Asp, and His would improve the identification of transmembrane regions is not immediately obvious. This chapter compares the membrane spanning segments of bacteriorhodopsin and the light subunit of the photoreaction center found in *Rhodopseudomonas viridis* determined using various hydrophobicity scales to examine the effect of discrepancies in certain residues. We have compared the values measured in Chapter 3 with values from Fauchere and Pliska (1983) to create a modified hydrophobicity scale.

METHODS

AMINO ACID HYDROPHOBICITY SCALES

The amino acid hydrophobicity scales of Fauchere and Pliska (F&P) and Engelman and coworkers (ESG) differ greatly in the hydrophobicity values for charged residues. A comparison of the prediction of the two scales will give information on the significance of these values in predicting membrane spanning segments. The hydrophobicity values obtained in the present study described in Chapter 3 are also included for the comparison to examine whether the results improve the prediction. Two scales are derived from the result in Chapter 3. Amalgam is the combination of the present result and the hydrophobicity of F&P and composed of values for Gly, Ala, Phe, Trp, Pro, Asp, Glu and His determined in the present study and the values for the rest of amino acids determined by Fauchere and Pliska (1983). The other scale ASP is derived from the calculation of the hydrophobicity values using the atomic solvation parameter approach as described below.

ASP has been derived using the atomic solvation parameter approach of Eisenberg and McLachlan (1986). The free energy of transfer of a molecule (ΔG) is approximated as the sum of atomic terms:

$$\Delta G = \sum \Delta\sigma_i A_i \qquad [1]$$

where σ_i is the atomic solvation parameter and A_i is the solvent accessible area of the atom. Equation [1] summarizes the idea that the atomic hydrophobicity is linearly related to the solvent accessible surface area of the atom and the linearity constant (atomic solvation parameter) differs depending on the character of each atom. The atomic solvation parameter of various atom are obtained from multiple regression analysis of experimental data using the following equation:

$$\Delta G = \Delta\sigma(C)\sum A(C_i, R) + \Delta\sigma(N/O)\sum A(N/O_i, R) + \Delta\sigma(O^-)\sum A(O^-_i, R)$$
$$+ \Delta\sigma(N^+)\sum A(N^+_i, R) + \Delta\sigma(S)\sum A(S_i, R) \qquad [2]$$

in which $\sigma(C)$ is the atomic solvation parameter for carbon and $A(C_i, R)$ is the solvent-accessible surface area of carbon atom i in a standard conformation of residue R (Shrake and Rupley, 1973), and so forth for the other terms.

The experimental data obtained in Chapter 3 and the accessible surface area of atoms (Shrake and Rupley, 1973) summarized in Table I were used to determine the $\Delta\sigma$ values for atoms. The multiple regression analysis was done by Apple Macintosh program STATWORKS. The result of analysis is compared with the result of Eisenberg and McLachlan (1986) obtained from the analysis of the data of Fauchere and Pliska (1983) (Table II). The predicted free energy of transfer of side chains calculated using $\Delta\sigma$ values in Table II and equation [2] is summarized in Table I.

The results for C and N/O are comparable. However the two results differ greatly for the $\Delta\sigma$ values of charged atoms. The number of data used in the present analysis is smaller than that of Fauchere and Pliska (12 vs. 20) and only one data point was used for N^+ (His). The predicted values for charged residues such as Arg, Lys may not be as convincing as those determined from experiments. However the correlation of the hydrophobicity scale obtained in this manner (ASP) with F&P ($R^2 = 0.75$) and ESG ($R^2 = 0.82$) was as good as any other scales described in Chapter 1 (see Table VI). The hydrophobic residues in ASP are about 0.5 to 1 Kcal/mol less hydrophobic and hydrophilic residues are 1 to 3 Kcal/mol more hydrophilic than the corresponding values in the F&P scale (see Table VII in Chapter 1).

Table I. Free energy of transfer determined by atomic solvation approach

residue	ΔG_{exp}(cal/mol)[a]	A(C)[b]	A(N/C)[b]	A(O-)[b]	A(N+)[b]	ΔG_{calc}(cal/mol)[c]	ΔG(cal/mol)[d]
Gly	-	-	-	-	-	0	0
Ala	-130	41	-	-	-	-533	-420
Phe	-2190	139	-	-	-	-1807	-2430
Trp	-2520	164	17	-	-	-2013	-3060
Pro	-290	73	-	-	-	-949	-980
His(n)[e]	-199	91	28	-	-	-987	-180
Asp(n)[e]	194	1	63	-	-	428	-
Glu(n)[e]	103	30	68	-	-	86	-
His(i)[f]	1950	91	-	-	28	1953	-
Asp(i)[f]	3810	1	32	41	-	3778	1050
Glu(i)[f]	3490	30	37	42	-	3523	870
Lys	-	92	-	-	41	3396	1350
Val	-	90	-	-	-	-1170	-1660
Leu	-	119	-	-	-	-1547	-2310
Ile	-	116	-	-	-	-1508	-2450
Arg	-	106	63	-	46	4215	1370
Asn	-	-5	82	-	-	639	810
Gln	-	21	89	-	-	350	300
Ser	-	13	31	-	-	48	50
Thr	-	-24	95	-	-	977	-350
Tyr	-	117	40	-	-	-1241	-1310

Table I (continued)

a. The free energy of transfer of side chains determined from octanol-water partition coefficient of tripeptides as described in Chapter 3.

b. Average areas exposed to solvent in Gly-X-Gly models for the unfolded state determined by Shrake and Rupley (1973). The area of α-carbon of Gly was subtracted from the total area of carbon atoms in other residues.

c. The free energy of transfer of side chains calculated according to the equation [2] using the atomic solvation parameter listed in Table II.

d. The free energy of transfer of side chains determined by Fauchere and Pliska (1983) (Table VII, Chapter 3). The free energy value for neutral form of histidine is assumed to be the same as that measurd at pH 7. The values for ionized form of Asp and Glu are assumed to be the same as those measured at pH 7.

e. Free energy of uncharged form of His was measured at pH 9 and that of Asp and Glu was determined at pH 2 as described in Chapter 3.

f. Free energy of ionized form of His was measured at pH 3 and that of Asp and Glu was determined at pH 10 as described in Chapter 3.

Table II Atomic solvation parameter ($\Delta\sigma$)

$\Delta\sigma$ (cal $Å^{-2}$ mol^{-1})	present result	Eisenberg and McLachlan(1986)
$\Delta\sigma$(C)	13.0 ± 2.2	16 ±2
$\Delta\sigma$(N/O)	-7.0 ± 5.6	-6 ± 4
$\Delta\sigma(N^+)$	-87 ± 10	-24 ± 10
$\Delta\sigma(O^-)$	-112 ± 20	-50 ± 9
$\Delta\sigma$(S)	-	21 ± 10

HYDROPHOBICITY PLOT

In the present study, BR and the bacterial photoreaction center (PRC) were chosen since the three dimensional structure of these two membrane proteins has been established with greater precision than any other membrane proteins (for review, see Rees et al, 1989; Popot et al, 1989). Four hydrophobicity scales described in the previous section are used and the result of Kyte and Doolittle scale (K&D) is from the literature (Kyte and Doolittle, 1982; Engelman et al., 1986; Michel et al., 1986). The average of hydrophobicity values of 20 amino acids in a segment was assigned to the residue in the center of the segment except for the analysis of BR using ESG scale. The latter was done in two ways. First, the average value was assigned to the N-terminal of a segment to compare the result of Engelman and coworkers (1986). Second the average value was assigned to the central residue of a segment and the result was compared with the first method. The averaged hydrophobicity value was then plotted from the N-terminal to C-terminal of protein. The hydrophobicity assignments to the sequence and averaging of the hydrophobicity in each window was done on a Apple Macintosh program Excel 2.2 using command macros. The results were plotted using Cricket graph 1.3 on a Macintosh.

RESULTS AND DISCUSSION

The topology of BR predicted by Engelman and coworkers is shown in Figure 2. Seven membrane spanning helices predicted by Engelman and coworkers (1986) are shown and part of the loop residing in the aqueous phase is supported by experimental evidence such as enzymatic cleavage or antigenic recognition. Those regions or residues are listed in Figure 2. The hydrophobicity plots of BR obtained using four different scales are shown in Figure 3. The midpoint line in these plots is the average of hydrophobicity

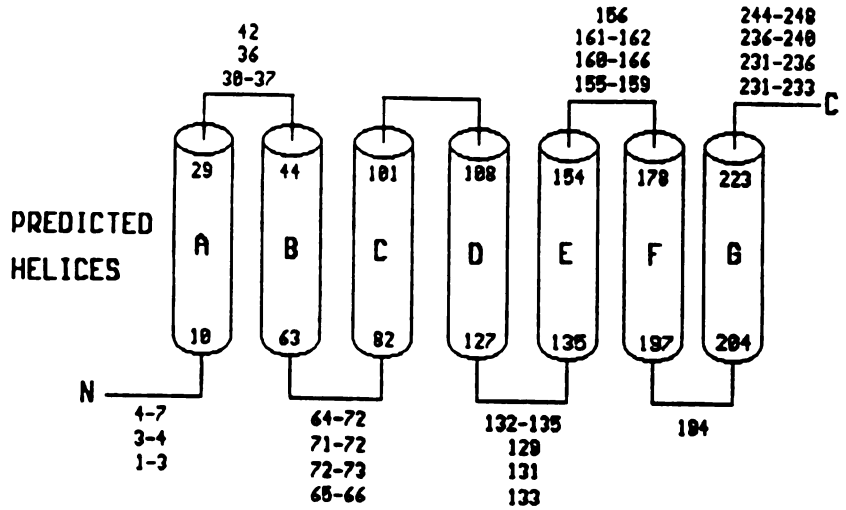


Figure 2. Topology of bacteriorhodopsin. Seven helices are predicted by Engelman and coworkers (1982). The numbers on the helices indicate the predicted end residues in transmembrane region and numbers outside indicate the regions or residues where experimental evidence indicate that they reside in the aqueous phase (Adapted from Engelman et al., 1986)

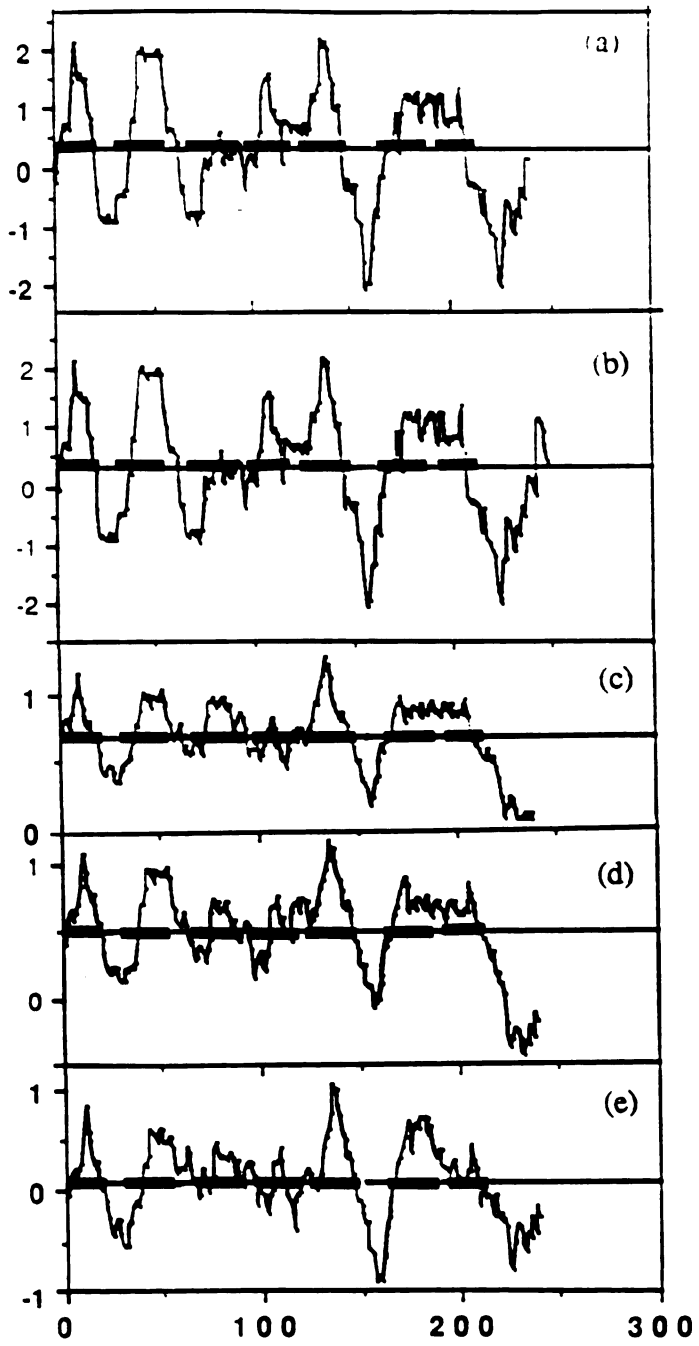


Figure 3. Hydrophobicity plots of bacteriorhodopsin using various scales; (a) ESG scale; (b) ESG scale; (c) F&P scale; (d) amalgam; (e) ASP scale. The average hydrophobicity of 20 amino residues in a segment was assigned as the hydrophobicity of central residue except in the plot (a) where the average of 20 residues was assigned to the first residue in the segment. The predicted region of transmembrane helix by Engelman and coworkers are shown as solid bars.

values of all the residues in the sequence. Figure 3-(a) and (b) were obtained using ESG scale. The average of 20 residue segment was assigned to the first residue of the segment (a) and to the central residue (b). The ends of the predicted segment differ by 10 residue depending on the way to assign the average of the segment and the features of hydrophobic clusters are the same. The plots of (c), (d) and (e) were obtained using F&P, Amalgam and ASP respectively. The helices, A, B and E are predicted in the same region of the sequence for all the scales. These helices contain no charged residues or one at the surface and are not sensitive to the scale chosen. The difference of about 2 Kcal/mol for Pro between F&P and ESG scales did not show any significant effect on the prediction.

The C helix predicted by the F&P, Amalgam and ASP scales was different from that of ESG scale. The C helix contains two Asp and one Arg. The free energy of transfer for these two residues in ESG scale is 10 Kcal/mol more hydrophilic than in F&P scale while those of Leu and Ala are comparable. Therefore the ESG scale would show a relatively insignificant hydrophobic peak for this region. If the midpoint line was chosen to be 0 instead of the average of hydrophobicity values of all the residues, the helix C would appear above the midpoint line in (a) and (b). The appropriate midpoint is crucial to predict the correct membrane spanning region. The average of hydrophobicity found in the sequence was chosen in this study to offset the effect of difference in absolute values among different scales. This approach can sometimes be inappropriate because marginally hydrophobic regions can be missed. The best criteria for the midpoint line are not, at present, clear.

There are subtle differences among the three scales, F&P, Amalgam and ASP. The area of helix C varied a little among the plots using the three scales. The short loop between the helices F and G is clear only in the plot using the Amalgam and ASP scales. The helices D and F are more prominent in plot (e) obtained using the ASP scale than in plot (c). The ASP scale is overall less hydrophobic than the F&P scale (Table I) and the difference in the prediction cannot be easily related to any particular difference in residues.

The hydropathy plot of the L subunit of PRC is compared with the transmembrane helices found by X-ray structural analysis in Figure 4 (Michel et al., 1986). The hydrophobicity plot using the four scales are shown in Figure 5. All of them show the five helices and predict the end regions of helices better than Kyte and Doolittle. The prediction is better than that for BR mainly due to the fact that PRC contains less charged residues than BR. However the end region of helix B where 5 among 10 residues are charged, was not predicted by any of the plots shown in Figure 5. Histidine is the only charged residue found in other helices and the difference of 3 Kcal/mol of the free energy of transfer for His among studies does not influence the result. The 22 residue long carboxy terminal of PRC contains 7 Trp residues (Michel et al., 1986). The K&D scale predicted the region under the midpoint line (Figure 4) because Trp is hydrophilic in the K&D scale. The F&P, amalgam and ASP scales show a hydrophobic peak in this region (Figure 5, b-d) and the ESG scale shows a small peak (Figure 5-a). In plot (d) obtained using the ASP scale, helix B is not as prominent as other helices and the loop between helices C and D is not as clear as in the other plots.

In summary, for membrane proteins of overall high hydrophobicity, the hydrophobicity value assigned to charged residues can influence the outcome of the moving segment approach. The present analysis shows that a 2 or 3 Kcal/mol difference per residue is not detected unless the residues are clustered in one region when the effect is amplified. A 10 Kcal/mol difference per residue is clearly detected in the hydrophobicity plot. Charge neutralization must play a role to accommodate several charged residues such as is found in helix B in PRC. The present methods cannot detect this particular type of membrane spanning region. This highlights an area that needs improvement. The position of the midpoint line also influenced the interpretation of the hydrophobicity plot. More general criteria for the midpoint line can only be obtained after more hydrophobicity plots of membrane proteins are determined.

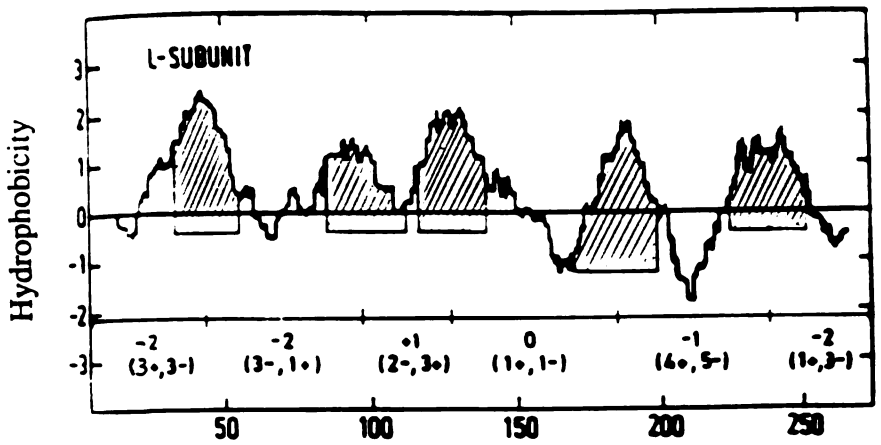


Figure 4. Hydropathy plot of the L subunit of photoreaction center found in *Rhodopseudomonas viridis* (Michel et al., 1986). Kyte and Doolittle algorithm was used with a window of 19 residue long and the average of a window was assigned to the 10th residue. The shaded area indicate the transmembrane helices determined by X-ray structural analysis. (Adapted from Michel et al., 1986)

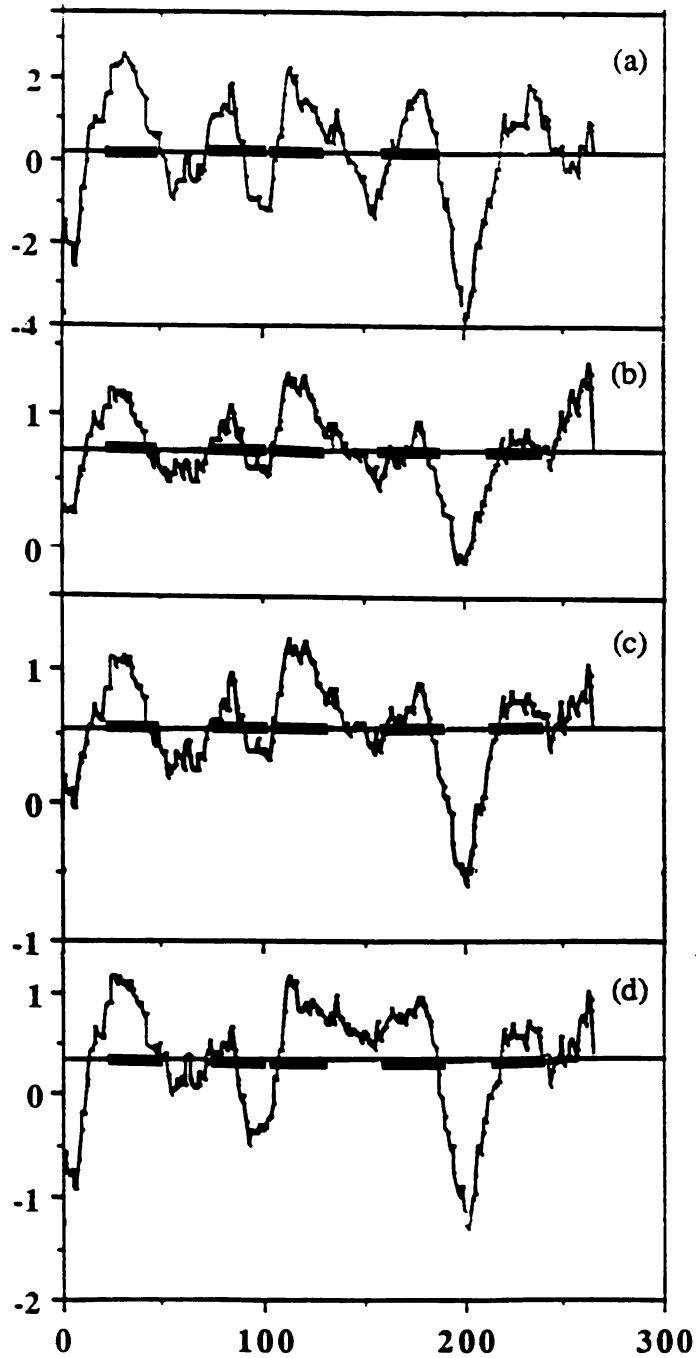


Figure 5. Hydrophobicity plot of the L subunit of photoreaction center using various scales; (a) ESG scale; (b) F&P scale; (c) amalgam; (d) ASP scale. A window of 19 residue long was used and the average of a window was assigned to the 10th residue. The transmembrane helix determined by Michel and coworkers (1986) are shown as solid bars.

CONCLUSIONS

A new hydrophobicity scale obtained using the atomic solvation parameter approach of Eisenberg and McLachlan (1986) differs significantly from that of Fauchere and Pliska. More analysis needs to be done to see whether this scale improves the prediction significantly. A 2 or 3 Kcal/mol difference of hydrophobicity of Asp, Glu or His did not significantly influence the prediction of membrane spanning segments of bacteriorhodopsin and bacterial photoreaction center protein, whereas a 10 Kcal/mol difference did. The discrepancy for the hydrophobicity of Trp among the various scales influenced the prediction when these residues were concentrated in one region of the sequence.

In conclusion, the membrane spanning helices of two proteins, bacteriorhodopsin and bacterial photoreaction center, are predicted reasonably well with all the hydrophobicity scales used in the present comparison.

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